THE MIGRATION OF LYMPHOCYTES TO NORMAL AND INFLAMED TISSUES IN THE RAT

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To those who appreciate the beauty of life

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CONTENTS

SUMMARY

Until two decades ago the fate and function of lymphocytes were largely unknown. The discovery that these cells were long-lived, showed unique mobility in migrating from the blood into the specialized lymphatic tissue of lymph nodes and spleen and repeatedly returning to the blood, and were immunologically competent, provided the basic answers to these questions. However, many aspects of the mechanisms involved in the initiation and propagation of immune reactions remained unclear. In particular it was not known to what extent lymphocytes entered other tissues and how, if at all, this behaviour was altered in sites of inflammation. It might be expected that immunologically active cells would enter non-lymphoid tissues as many immune reactions are implemented at least in part in these peripheral sites; also direct examination of afferent lymph draining non-lymphoid tissue has detected some lymphocyte traffic. The studies reported here were designed to provide quantitative estimates of lymphocyte migration into non-lymphoid tissues under normal and pathological conditions and attempts were made to define the kinetics of this flow of cells and to investigate some aspects of the control mechanisms that may regulate it.

To this end the basic techniques of marking rat thoracic duct lymphocytes and tracing their distribution in syngeneic recipients, that allowed Gowans and his colleagues to establish the concept of the recirculating pool of small lymphocytes, were used. Critical evaluation of different labelling methods was essential, as great sensitivity was required in order to detect accurately the small concentrations of labelled cells expected in non-lymphoid tissues.

Radioisotopic labelling of lymphocytes would allow

estimation of their tissue distribution after iv transfer by assay of total tissue radioactivity provided the label remained attached to the cells and these cells were extravascular. The contamination duct to intravascular labelled cells was minimized by perfusing a large volume of saline through the whole circulatory system before sampling the tissues. An indirect measure of the extent of loss of isotope from the injected cells was made by assaying the activity in many compartments particularly those free from cells such as plasma, urine and the perfusion washout fluid. Examination of the latter also provided information about widespread contamination of tissues by freely diffusible isotopes and was important in assessing the usefulness of particular labelling compounds for the purpose of tracing lymphocytes to areas of low cell concentration.

A comparison of the distribution of activity after labelling lymphocytes with many isotopes showed serious limitations in the use of some 'labels' but suggested that the results obtained using ^{51}Cr were most likely to provide realistic estimates of labelled cell content of tissues. Ranges for lymphocyte cell content and flux (10^4 - 10^5 lymphocytes/gm/hr) in non-lymphoid tissues were derived from kinetic distribution studies using multiple recipients of the same labelled cell population and examining groups of recipients at intervals after cell transfer. The estimates were in the same range as those derived from data on afferent lymph cellular flow obtained by direct measurements in other species such as sheep and pigs. The time taken for labelled cells to traverse non-lymphoid tissues while not measurable precisely was probably shorter than that taken to enter and leave either spleen (5-6 hrs) or lymph nodes (18 hrs).

Migration of lymphocytes into inflammatory lesions in the skin was found to be increased and usually the transit time remained rapid. Exposure of lymphocytes to a low

dose of trypsin in vitro drastically reduces their efficient migration into lymph nodes: it was found that such treated cells also failed to migrate in increased numbers into lesions in the skin but entry into normal tissues was unaffected. It was suggested that in both lymph nodes and cell-mediated immune lesions the enzyme treatment in some way interfered with the lymphocyte-endothelial cell interaction; this implied there was a change in the inflamed venular endothelium that allowed a more efficient clearance of lymphocytes from the blood. It was further suggested that the highly specialized function of lymph node post-capillary venular endothelium that is associated with a morphological specialization may be an adaptation of a temporary functional change that occurs in venular endothelium in cell-mediated immune lesions because this latter type of lesion is widespread even in invertebrate animals but lymph nodes are very recent developments in phylogenetic terms.

Using the same techniques of following labelled cells supplemented by autoradiography of recipients' tissues the migration of thoracic duct lymphocytes to the 'primary' lymphoid organs was studied. The thymus and bone marrow are both sites of very active lymphocytopoiesis which is mainly independent of antigenic stimulation. Both have been regarded as insignificant compartments of the recirculating lymphocyte pool. However, the thymus was found to contain a greater concentration of radioactivity than all other non-lymphoid tissues except liver and kidney and contained approximately 0.1% of the total recirculating pool at equilibrium. The kinetic distribution curve was similar to that for lymph nodes suggesting a modal transit time of at least eighteen hours. Autoradiography showed that the migrating cells were limited to the thymic medulla.

The bone marrow was found to accept up to one quarter of an injected 'bolus' of labelled lymphocytes. These cells

were extravascular and later returned to the blood somewhat more rapidly than the lymphocytes that entered the spleen. The existence and size of this migration pathway was confirmed using a functional indicator of lymphocytes and the capability of the bone marrow to act as a 'secondary' lymphoid organ was examined. It was shown that this tissue could concentrate particulate antigen from the blood, and support antigen-induced transformation and proliferation of lymphocytes and these findings taken with the high flux of immunocompetent lymphocytes suggested that this organ could function in the initiation and propagation of immune responses.

CHAPTER ONE

General Introduction

Lymphocytes have been defined by several criteria. By morphology under the light microscope mammalian lymphocytes are mostly round cells of less than eight microns diameter with a round nucleus and a thin rim of cytoplasm. Such cells are the main cellular constituent of lymph in the major lymphatic ducts and are also present in high concentrations in lymph nodes and the white pulp of the spleen. The thymus contains large numbers of these cells which can also be found in discreet aggregations in the gut-associated lymphoid tissues and similar structures in other parts of the body such as the nodules found in a subepithelial position at intervals along the bronchi. The blood contains significant numbers of lymphocytes as does the bone marrow. In addition cells with lymphocyte morphology can be identified in low concentration scattered through most tissues and more concentrated aggregates occur in chronic inflammatory lesions such as the granulomata of tuberculosis.

Since the demonstration that lymphocytes have an immunological function and are essential for the development of specific activity to antigens by either a humoral response - ie the production of antibody molecules - or a cell mediated response as in delayed type hypersensitivity or homograft rejection, much work has been carried out to determine the precise mechanisms involved in such reactions. This together with studies investigating the origin and fate of lymphocytes has given rise to some generally accepted notions about the role of lymphocytes in immune responses.

This simplified summary of these ideas does not include a

comprehensive review of the evidence that has been produced for each point.

In the mammal and particularly in rodents in which most of this work has been done, lymphocytes are produced by division and maturation of haemopoietic pluripotent stem cells (Micklem et al 1966). In the embryo this occurs in the yolk sac blood islands, in the foetus in the liver and splenic red pulp, and in the adult mainly in the bone marrow. Such immature lymphocytes comprise at least two populations, one of which migrates and undergoes further maturation and division in the thymus to give rise to T cells (Miller 1966, Wolstenhdme & Porter rev.1966), while the other undergoes maturation without thymic influence to B cells. In birds such as the chicken this latter maturation is under the influence of the Bursa of Fabricius (Chang et al 1955), but in rodents mainly occurs in the liver of the foetus (Owen et al 1974) and in the bone marrow of adults (Osmond & Nossal 1974 a & b) although this latter micro-environment is not essential (Kincade et al 1975, Rozing et al 1976). There are therefore at least two populations of lymphocytes both of which are believed to contain cells that have specific receptors for particular antigens (Hammerling & McDevitt 1976, Makela 1970).

The blood lymphocyte content remains remarkably constant despite the entry of enough cells into this compartment both from the major lymphatic ducts and by new cell production in the primary lymphoid organs - bone marrow and thymus (Everett et al 1964) to replace the total blood population several times each day (Mann & Higgins 1950; rev.Yoffey & Courtice 1956).

This posed the question of the fate of blood lymphocytes. The use of radioisotopes allowed rigorous proof of the idea already suggested by circumstantial evidence that many lymphocytes were long-lived and continuously recirculated in high numbers between the blood and secondary lymphoid

organs - the lymph nodes and spleen (Gowans 1957 a & b, 1958, 1959; Gowans & Knight 1964; Everett et al 1964).

It has also been suggested that most if not all long-lived recirculating lymphocytes have been derived by clonal expansion of lymphocytes that have reacted specifically with antigen (Greaves et al rev.1974, Sprent rev.1977). 'Virgin' B lymphocytes at least, which are competent to react to antigen, but have not yet done so probably have a short life span in the absence of such specific interaction and are thus part of a population that is being replaced continuously (Strober 1970). The distribution and fate of such cells is less clear. However the important points that emerge are that lymphocytes are highly motile cells that have the ability to bind and react with antigen.

These properties must be correlated with the ability of the animal to react immunologically to an antigenic stimulus. Most natural external antigens gain access to the body across the skin or alimentary or respiratory epithelium rather than by direct invasion of the blood or secondary lymphoid organs yet it has been amply proved that these latter organs are important in the development of immune responses. The response may result in the production of effector function that in turn results in inflammation at the peripheral site of antigen entry.

Although antibody molecules could be distributed from lymph nodes or spleen to the periphery without movement of cells the latter is obligatory for the development of cell mediated immune reactions. The initial sensitization of the animal to antigen could depend entirely upon movement of antigen to the secondary lymphoid organs and although such movement is known to occur it has also been shown that sensitization of lymphocytes can occur at the periphery (Macher & Chase 1969, de Sousa & Parrott 1969). In fact it is likely that this must be so as lymph nodes are phylogenetically recent and animals that have no such structures produce effective immune responses.

There is thus strong circumstantial evidence to suggest that lymphocytes migrate through all tissues in order to permit senzitization and effector functions. Direct evidence of such migration has been obtained by draining the contents of afferent lymphatic vessels in large animals where such procedures are feasible. In the sheep, for example, the cell output from such vessels was very much lower than that of efferent lymphatics (Smith et al 1970a) confirming the idea that most of the efferent duct cell content was derived directly from the blood within the lymph nodes but this large discrepancy in total cell output figures has tended to de-emphasize the possible functional importance of the blood - afferent lymphatic - lymph node migration pathway.

In simplified terms the immune response can be considered as a reflex arc initiated by an antigenic stimulus applied at the periphery. There follows a complex interaction between antigen, both T and B lymphocytes and macrophages all of which can be mobile. The secondary lymphoid organs provide the most efficient site for this interaction which has many similarities to a synaptic junction including both suppressive influences and a memory factor supplied by the high volume lymphocyte flux using the direct blood-lymph node or spleen pathway of recirculation. The blood distributes the effector function either as cells or antibody or both to all tissues including the original site of antigen entry and allows an inflammatory response to develop which in general is appropriate in time and situation.

The aim of this project was therefore to attempt to quantify the migratory flux of lymphocytes through non-lymphoid tissues under both normal and pathological conditions in order to gain insight into the relative importance of this migratory route and also to look for factors involved in the control of inflammatory lesions which involve lymphocytes.

The results to be described also throw some light on the extent of lymphocyte migration through the primary lymphoid

organs and suggest that these organs, as well as being sites of antigen-independent lymphopoiesis, also have some of the functions which have been conceived to be exclusive to secondary lymphoid organs.

The hypothesis of the recirculating lymphocyte pool arose from the work on the migration of small lymphocytes mainly through the lymph nodes (Gowans 1959) and spleen (Ford 1969a). This pool comprises a multicompartmental system with interchange of lymphocytes between some of the compartments at varying rates. This type of model can be studied by standard tracer techniques and the kinetics of distribution between the compartments can be determined if marked members of the mobile population are introduced into one compartment and other compartments are sampled after various time intervals. Interpretation of this type of data is valid if the mobile population is homogeneous and its constituent members act independently.

As far as lymphocytes are concerned this requires some method of identification of cells introduced into an animal which must also accept the 'marked cells' as normal. Available markers for isogeneic or syngeneic lymphocytes vary in their suitability for different purposes. Chromosome abberations that are not lethal or toxic allow 'normal' cells or their descendants to be followed for long periods provided that preparations of dividing cells are available to allow identification. Functional and antigenic markers allow the study of sub-populations of cells that may not be morphologically identifiable. Radioactive isotopes can allow positive identification of labelled cells in autoradiographic preparations of tissue sections and within limitations can also allow indirect scoring of cells in tissues by measurement of total tissue radioactivity.

For this project a compromise between purity of cell type in the population under study, known physiological migratory capacity and ease of availability of large numbers of lymphocytes was achieved by using thoracic duct lympho-

cytes (TDL) obtained from inbred strains of rats. For most of the work such cells were labelled with various radioactive isotopes and introduced into the blood of syngeneic recipients which were examined after various time intervals.

CHAPTER TWO

General Materials and Methods

1 Cell suspension media, fluids for injection, etc.

(i) PBS — Dulbecco's phosphate buffered saline (Oxoid, Code BR 14a) Tablets dissolved in distilled water and solution autoclaved and then stored at $4^{o}C$.

(ii) DAB — Dulbecco's sterile PBS with added mineral salts (Ox_oid, Code SR 39a). Stored at $4^{o}C$.

(iii) DAB- 1) DAB-20) — DAB with added Heparin (Evans Medical, Speke, Liverpool, GB) to a final dilution of 1 or 20 units/ml.

(iv) RPM1-1640 — Cell culture medium Rosewell Park Memorial Institute 1640 with added L Glutamine (Gibco-Biocult, Irvine, Scotland) stored sterile at $4^{o}C$.

(v) FCS — Foetal calf serum (Gibco-Biocult, Irvine, Scotland). Sterile aliquots of 10ml stored at $-20^{o}C$. Thawed before use.

(vi) Ficoll — Pharmacia Fine Chemicals, Upsalla, Sweden. 14% in distilled H_2O.

(vii) Hypaque — 45% Sodium diatrozoate (Winthrop Labs, Surbiton, GB).

(viii) EDTA — Ethylenediaminetetraacetic acid. Either di- or tetra- sodium salts. (BDH, Poole, GB).

2 Animals

Several strains of closely inbred rats bred and maintained in the departmental colony were used. F_1 hybrids between some of the inbred strains were also used. For individual experiments animals of similar weight but either sex were used and cell transfer was between syngeneic donors and recipients unless otherwise stated.

TABLE 2.1

Rat Strain	Major Histocompatability Antigen
AO	2
PVG/c	5
DA	4
AS	1
HO.B2	2

AO on PVG/c background congenic strain.

3 Surgical Procedures

(i) Thoracic Duct Cannulation

The method used was that described by Ford & Hunt (Handbook of Experimental Immunology 2nd ed.1972, Ed.Weir), which is Gowans' modification of the technique originally described by Bollman et. al. (1948). The plastic cannula (PP50 Portex Ltd) was preformed by mild heat and filled before insertion with DAB-20 to help prevent clotting while lymph flow was being established.

Cannulated rats were maintained in barred restraining cages (Bollman 1948). An intravenous infusion was administered via a plastic cannula and hubless 23g needle inserted into a lateral tail vein and held in place with adhesive tape which also served to splint the tail. After an initial dose of 1.0ml of DAB-20, DAB-1 was infused at 2-3ml/hr via a roller pump.

Lymph was collected into sterile 100ml glass conical flasks containing 5ml of DAB-20 as anti-coagulant. The flasks were kept on ice and collections of up to 16 hrs were made before renewing the flask.

(ii) Cell Injections

All injections were carried out under light ether anaesthesia. Intravenous (iv) injections of cell suspensions in PBS, DAB or RPMI-1640 were into a lateral tail vein via a 23g or 25g needle. Up to 2ml was routinely injected over a few seconds with no adverse effect on any recipient.

(iii) Whole Body Perfusion

This technique was developed in an attempt to remove most intravascular cells from the body. It also yielded important information relating to the distribution of cell-free radioactivity with some of the radioisotopes.

The recipient was placed under deep ether anaesthesia and a cardiac blood sample was obtained (usually 5ml) via a 21g needle introduced through the diaphragm into the apex of the heart.

Immediately afterwards the rat's neck was dislocated and the chest opened so that the sternum was separated from the ribs on either side, reflected upon the sternomanubrial joint and pinned to the cork dissecting board. The thymus was dissected free of the major vessels in the mediastinum and a linen suture was passed around the ascending aorta. A plastic cannula (PP90 Portex Ltd) filled with DAB-20 was then inserted through the apex of the left ventricle into the root of the aorta where it was secured by the ligature. This procedure was carried out as fast as possible to minimize vascular reactions to the inevitable anoxia.

A large volume of saline was then introduced into the arterial circulation after the inferior vena cava in the thorax and the right and left atria had been severed to allow escape of the perfusate into the thoracic cavity. The perfusate fluid was collected by means of a suction pump. The perfusion was done by gravity feed or by syringe and routinely 250ml of DAB-20 and PBS was introduced over about ten minutes. The effluent perfusate though frankly bloody to begin with very rapidly became clear and most perfused tissues became obviously blanched. Patchy perfusion was often encountered in the kidneys especially if any delay was present during the establishment of perfusion. This was presumed to be due to vascular spasm.

The lungs were perfused directly via a cannula introduced through the right ventricle into the pulmonary artery though this was technically more difficult than for the aorta. Because it was almost impossible to suture the cannula in place without tearing the pulmonary artery, the cannula was held in place for a short time with forceps and the perfusion prolonged just enough to effect maximum blanching of the lungs. There was usually a small degree of patchy petechial haemorrhage in these organs.

For tissue fixation after preliminary perfusion with DAB-20, 1% Gluteraldehyde was introduced into the arterial circulation resulting in marked muscle spasm and rapid fixation of most tissues.

4 Cell Preparation and Counting

Routine counts of thoracic duct cells were made in a Coulter Electronic Particle Counter with suitable dilutions of lymph or cell suspensions in Isoton diluent. The threshold was set to avoid counting the few contaminating erythrocytes which have significantly

smaller cell volume. For peripheral blood the diluted suspension was treated with Zaponin and counted in the Coulter counter or after treatment with acetic acid and either Leishman or Toluidine Blue stain, counts were made in Neubauer improved haemocytometer chambers viewed with transmitted light with x 10 or x 25 objective lenses.

Viability of lymphocyte suspensions was assessed by counting the proportion of cells that successfully excluded 0.2% Trypan Blue at room temperature over a period of 5 minutes.

5 Dissection

Weighed carcasses were carefully dissected and individual organs were separated cleanly from adjacent structures, removed, weighed and processed. Heparinized blood samples were divided; 2.0ml were diluted with 8.0ml PBS and layered on 3.0ml of Ficoll (67ml 14%) / Hypaque (20ml 45%) mixture, centrifuged at 1200g for 30 mins at 15°C and the interface layer collected for assay of blood leukocytes. A further 2.0ml was centrifuged at 1500g for 10 mins to obtain plasma.

6 Histology

(i) Fixatives

a Cacodylate Buffered Glutaraldehyde 1%

Glutaraldehyde (TAAB Labs, Reading) 25%	3ml
Distilled water	40ml
Buffer (Sodium cacodylate 42.8g (0.2M) 1 N HCl 6.9ml, distilled water to 1000ml)	30ml

This was used for all perfusion fixation and also for post-perfusion immersion fixation.

b Neutral Formal Saline was used as an alternative immersion fixative for non-perfused tissues.

(ii) Processing and Tissue Sectioning

Fixed tissues were processed for paraffin embedding

and microtomy by standard procedures, and sections were cut at 5μ and either stained with Haematoxylin and Eosin or 'dipped' for autoradiography.

Occasional tissues were examined after cutting sections of tissue, frozen with solid CO_2, in a freezing microtome at -20°C.

7 Radio Assay

(i) Autoradiography

Dewaxed slides or washed smears were dipped in freshly prepared emulsion warmed to 42°C in the dark. The emulsion was Ilford G5 diluted 2:1 with distilled water with addition of glycerol to give a final concentration of 1%. The dipped slides were allowed to dry in air after placing on draining racks at an angle of about 60°. When absolutely dry the slides were placed in light-tight plastic boxes (Clay-Adams Inc) at 4°C to allow exposure. After exposure for usually 2-6 weeks the emulsion was developed with Kodak D-19 developer for 5-10 minutes at 20°C in total darkness. After rapid and thorough rinsing the emulsion was fixed with Kodak 'Metafix' for 10 minutes and then washed again.

Sections were stained through the emulsion with either Methyl Green and Pyronin or Haematoxylin and Eosin.

(ii) Assay of β-radioactivity by Liquid Scintillation Counting

The method described by Ford & Hunt (Handbook of Immunology Ch.23, 2nd ed.1973, Ed.D M Weir) was used with one important modification. The use of Hydrogen Peroxide (100 vols) to bleach pigment in the digested samples and therefore reduce colour quenching was found to cause spurious counts in the low energy range (narrow ^3H window), particularly when clear fluids were examined.

A series of experiments showed that these artefactual counts were due to the H_2O_2 presumably following chemiluminescence of nascent oxygen from unreacted H_2O_2. Several methods of attempting to remove excess H_2O_2 (incubation at 50°C for 2 hrs, addition of the enzyme catalase, shaking and prolonging the interval between addition of H_2O_2 and the scintillant mixture) were only partially successful and the most efficient method was found to be the addition of 2% normal rat erythrocytes to the sodium hydroxide used for the original digestion of all samples except those already heavily contaminated with blood. This allowed maintenance of a standard treatment for all samples.

Table 2.2 shows data extracted from an experiment in which samples of non-active and tritium containing fluids were treated with digestion, with and without H_2O_2 and compared with the same fluids counted without any treatment except the addition of scintillant. The H_2O_2 was added 48 hrs before the scintillant and the vials were then allowed to stand for 12 hrs before counting. Counts per minute over 10 mins for each sample were then measured at 12 hr intervals for 4 days for both Narrow and Broad ^3H channels. The external standards ratio (ESR) value gives a measure of the quenching for each sample and as usual this value falls slightly with time for all samples. It is clear that the samples with added H_2O_2 but no means of reacting it produce a large excess of counts and that the major proportion of such counts is in the Narrow ^3H range, whereas true background counts are distributed with approximately half in the Narrow ^3H range, and true ^3H counts with approximately two thirds in this range.

TABLE 2.2

Time of counting after addition of scintillant	COUNTS PER MINUTE											
	NO DIGESTION				DIGESTION ONLY				DIGESTION + H_2O_2 *			
	.5ml cont			.5ml ^3H			.5ml cont			.5ml ^3H		
	N^3H[a]	B^3H[b]	ESR	N^3H	B^3H	ESR	N^3H	B^3H	ESR	N^3H	B^3H	ESR	N^3H	B^3H	ESR	N^3H	B^3H	ESR
12 hrs	41	52	.464	125	186	.457	14	27	.409	102	160	.375	1336	1358	.385	698	700	.389
4 dys	8	18	.432	95	158	.428	10	23	.380	93	155	.343	343	363	.307	330	391	.315

* 48 hrs before scintillant
a) Narrow tritium channel
b) Broad tritium channel

Counting was done in a Beckman LS 250 dual channel system with automatic quench correction and external standard ratio facilities which allowed simultaneous counting of ^{3}H and ^{14}C where necessary. Disintegrations/min were calculated from counts/min with reference to calibration coefficients and degree of spillover between channels.

(iii) Assay of γ-radioactivity with deep-well γ scintillation spectrometer

Weighed tissue or fluid samples were assayed without processing in capped plastic tubes in an LKB (Wallac) Ultragamma 1250 dual channel γ ray spectrometer with a 2 x 2" thallium-activated sodium iodide crystal. Pulse height analyser facilities for each channel and automatic comparison of counts in each channel with reference samples allowed discrimination between isotopes when necessary. Counts/min were not converted to dpm for γ emitters but were related to a standard sample for each experiment.

Counts of both β and γ activity were accumulated to give a standard deviation (SD) about the mean count rate of under 10% and in most cases well below 1%. Count rates less than 2 SD above mean background levels were discarded.

CHAPTER THREE

A Comparison of Radioisotopic Cell Markers for Studying Lymphocyte Migration in vivo

Introduction

General considerations of using tracer methods for investigating cell migration have been discussed in the General Introduction. The choice of a marker depends on the particular purpose for which it is intended. Low energy β-emitting radionuclides are preferable for high resolution autoradiography. However, γ-emitters have the advantage of being detectable at greater distances from their source and therefore they avoid the necessity of complex digestion and counting procedures.

The ideal label for cell marking is one which is efficiently taken up and bound to intracellular constituents without being toxic or disturbing the normal function of the cell. It should also be readily detectable and remain with the cell as long as the latter is alive. Such a compound does not exist and thus compromises must be made according to particular circumstances.

For the study of lymphocyte migration, particularly to non-lymphoid tissues where total tissue radioactivity should reflect viable cell content - ie the indirect scoring of cells - the most important feature about a marker is that any label that is not associated with cells should not contaminate the tissues. Thus a degree of elution from the labelled cells could be tolerated if such free label was rapidly excreted rather than re-utilized. This type of factor is much less critical in situations where direct scoring of marked cells is feasible - eg chromosome or autoradiographic analysis of cells in smears

where the scoring of cells as labelled depends on a positive association of cell and marker so that with radiolabels as long as enough activity remains with the cell to develop the autoradiographic grain the cell will be scored and grains not related to cells are ignored.

The type of radioactive compounds available for labelling lymphocytes fall into three categories:

(i) Physiological metabolic precursor molecules with one or more atoms of the radioisotopic form. These are for practical purposes indistinguishable chemically or biologically from the non-active molecule and are least likely to be chemically toxic if used in physiological concentrations. Examples are tritiated or carbon labelled amino-acids or nucleotides.

(ii) Analogues of compounds that would otherwise be included in Category (i) but containing a radioactive atom that replaces a non-active atom of different species which does not interfere with the metabolism of the compound but may introduce an extra element of chemical toxicity - eg Selenium-75 substituted for sulphur in methionine or ^{125}I attached to deoxyuridine that will compete with thymidine for incorporation into deoxyribonucleic acid (DNA).

(iii) Unphysiological radioactive compounds which may bind in an unselective way to intracellular constituents and may well be chemically toxic - eg ^{57}Cr, ^{99m}Tc, and ^{111}In.

All the above categories may be inherently toxic to cells because of the radioactivity and such radiotoxicity depends upon the site of incorporation of the radionuclide together with the range of emitted energy and the dose - ie the cellular specific activity.

Against this background several compounds that have been used for radiolabelling lymphocytes were investigated for their suitability for tracing such labelled cells into non-lymphoid tissues in the following general experimental model.

Thoracic duct lymphocytes were 'labelled' either in vivo with ^{3}H Thymidine or most commonly in vitro culture with one of a variety of compounds. After thorough washing to remove unbound label the cells were injected intravenously into syngeneic recipients which were killed 24 hrs later. At the time of death the blood was sampled and then the vasculature 'perfused' with a large volume of PBS to remove intravascular label (see Methods) before tissues were excised. Weighed samples of the tissues together with samples of body fluids and the original injection suspension were assayed for total radioactivity by scintillation counting of either β or γ emissions.

Some experiments utilized recipients with indwelling thoracic duct cannulae to monitor the passage of label into the lymph both associated with the cells and in the lymph plasma.

Urine was collected semiquantitatively by collection of the output from restrained rats without catheterization of the bladder.

Methods

Table 3.1 summarizes the radiolabels studied with respect to their energy of emission, category as defined in the introduction, source of supply, labelling dose and approximate uptake efficiency - ie the proportion of the total activity added to the culture that remains with the cells in the final injection suspension.

TABLE 3.1

Lymphocyte Radiolabels

Category	Radionuclide[a]	Specific Activity	Code No.	Half-life & energy emission	Dose[c] /ml	% uptake into cells in culture
(i)	L-(4,5-^{3}H) Leucine	55 Ci/mMol	TRK 170	β 12 yr	10 μCi	$<$ 10
(i)	L-(U-^{14}C) Leucine	270 mCi/mMol	CFB 67	β 5760 yr	1 "	$<$ 10
(i)	(5-^{3}H) Uridine	25 Ci/mMol	TRA 178	β 12 yr	10 "	$<$ 10
(i)	(2-^{14}C) Uridine	62 mCi/mMol	CFA 315	β 5760 yr	1 "	$<$ 10
(i)	(6-^{3}H) Thymidine	5 Ci/mMol	TRA 61	β 12 yr	1 uCi/g body wt/dy	?
(ii)	(5-^{125}I) Iodo-deoxyuridine	100 mCi/mg	IM 352	γ 60 dys	.5-1.0 uCi	$<$ 10
(ii)	^{75}Se-L-Selenomethionine(b)	70 mCi/mg	SEMM-1	γ 160 dys	3.3 "	$<$ 10
(iii)	$Na_2\ ^{51}Cr O_4$	50-400μCi/mg	CJS 1P	γ 28 dys	10 "	$\simeq$ 2
(iii)	$Na_2\ ^{99m}Tc\ O_4$ (from generator)	variable	MCC 3	γ 6 hrs	up to 3 "	$\simeq$ 2
(iii)	^{111}In-8-hydroxyquinolone	carrier-free	INS 1	γ 67 hrs	0.1[d] "	$>$ 85

(a) All supplied by Radiochemical Centre, Amersham, except (b).
(b) Supplied by CIS via British agents - Eurotope Services Ltd, 104 East Barnet Rd, New Barnet, Herts.
(c) All in vitro cultures were in medium RPMI 1640 with L glutamine + 10% FCS at 5 x 10^{7}-10^{8} cells/ml for 1 hr at 37^{o}C except (d).
(d) Indium-oxine uptake was in normal saline at 10^{8}cells/ml for 10 mins at room temperature.

Thoracic duct lymphocytes were removed from lymph by centrifugation and for Category (iii) labels were further spun over Ficoll/Hypaque to remove most contaminating erythrocytes before suspension in tissue culture medium for labelling.

After labelling the cells were washed three times by centrifugation out of 40ml volumes of PBS and finally spun through 5ml of 50% FCS/PBS to exchange any surface adherent label. The cell pellet was then resuspended to a suitable volume and concentration for injection.

Two exceptions to this general method were used:

(a) ^{3}H Tdr was administered to growing rats at a dose of 1 μCi/g/dy i.p. for a period of three weeks to label all cells including lymphocytes that were synthesizing DNA during this period. A further three weeks was allowed to elapse before labelled TDL were collected by the establishment of a thoracic duct fistula. This was to allow recently formed cells time to either dilute out their label by further cell division or to die if they had a lifespan of less than three weeks. Thus the label in the TDL collected during the 48 hrs after cannulation was restricted to a population of relatively long-lived recirculating lymphocytes. Such cells were washed once after collection before resuspension for injection.

(b) ^{111}In-Oxine binds avidly to protein and therefore cells were labelled in protein free solutions. Indium-111 was recently developed as a cell label by M L Thakur and colleagues with particular application to polymorphonuclear leucocytes and platelets because it has good physical characteristics for external detection and a suitable half-life for studies in patients.

The few experiments summarized here were done with Dr Thakur and were designed to determine whether

lymphocytes would be labelled and would migrate normally and also to compare the tissue distribution in non-lymphoid tissues with the other labels.

The ^{111}In was chelated with 8-hydroxyquinolone (oxine) by addition of 50µl-150µl of 1mg/ml solution of oxine in ethanol to Indium chloride solution previously buffered to pH 5-6 by the addition of approximately 200µl of .3M acetate buffer. The complex which formed rapidly at room temperature was extracted twice in equal volumes of chloroform and the latter evaporated off in a boiling water bath. The residue which contained about 90% of the original activity was redissolved and stored in a small volume of ethanol. Before addition to cell suspensions the required activity was removed and the alcohol diluted in four volumes of normal saline.

Pilot experiments showed that uptake was most efficient in protein free normal saline at a cell concentration of greater than 50 x 10^6ml. Labelling was efficient at room temperature as well as 37°C but was slower at 4°C. (Table 3.2). Dilution of the cell suspension also reduced the labelling efficiency (Table 3.3), as did the use of culture medium (Table 3.4), probably because components of the latter competed for the binding of ^{111}In and broke the relatively weak oxine complex. This mechanism probably underlies the intracellular binding of Indium for the oxine does not remain within the cell. The complex being lipid-soluble serves to carry the oxine into the cell where binding occurs because of the greater avidity of as yet unknown cytoplasmic molecules (Thakur et al 1977).

TABLE 3.2

The effect of temperature on ^{111}In-oxine uptake into TDL in normal saline at 50 x 10^6/ml.

	% uptake	
Culture time (mins)	37°C	4°C
5	95	37
15	96	60
30	98	75
60	98	80
90	98	90

TABLE 3.3

The effect of cell concentration on the efficiency of ^{111}In uptake in normal saline for 5 mins at 37°C.

Cell concentration x 10^6ml	% of uptake at 70 x 10^6/ml
70	100
55	93
42	84
28	78
14	38

TABLE 3.4

The effect of culture medium on labelling efficiency of ^{111}In-oxine for 5 mins at 37°C.

Medium	% uptake
Normal saline	98
Phosphate buffered saline	95
RPMI 1640	60

After labelling with Indium-111 cells were thoroughly washed as for the other labels including the final spin through 50% serum to exchange any contaminating surface protein which was labelled.

Recipients received cell doses of between 10^7 and 2×10^9 in a volume of 2.0ml via the lateral tail vein. The cell number was not found to alter the proportional distribution and enough cells were injected to carry the requisite radioactivity for counting.

In some experiments labelled TDL were heat-killed by culturing for 30 mins in PBS at $56^{\circ}C$ after which time more than 95% of the cells failed to exclude Trypan Blue but remained intact.

Results

Table 3.5 summarizes results pooled from many experiments. Not all the parameters were measured in each recipient and the results are standardized by expressing them as the percentage of injected activity per whole organ or per total volume of body fluid. The original data were computed as % injected dose per gram of tissue from weighed aliquots and totals were derived from organ weights extrapolated from those measured after complete dissection of several rats. These weight and volume factors for a 200g rat are given in the first column of the tables.

TABLE 3.5 FOOTNOTES

1. Standard deviation - percentage of arithmetic mean.
2. Weight or volume factor by which %/gm or %/ml multipled to give whole organ or fluid value in a "standard" 200 gm rat.
3. n = number of recipients.
4. Recipients of ^{99m}Tc-labelled TDLs - non-perfused.
5. ^{3}H-Tdr - cells labelled in vivo.
6. ( ) number of recipients when different from n.
7. Small intestine avoiding Peyers Patches.
8. - = not sampled.
9. Separated on Ficoll/Hypaque.
10. Total for all tissues and fluids.
11. Approximate.

TABLE 3.5a

PERCENTAGE INJECTED DOSE PER WHOLE ORGAN 24 HRS AFTER I.V. TRANSFUSION OF LABELLED SYNGENEIC THORACIC DUCT LYMPHOCYTES ± S.D.[1] TISSUES SAMPLED AFTER WHOLE BODY PERFUSION.

LYMPHOID TISSUES

SSUE	W.F.[2]	ISOTOPE n[3]	^{3}H-Udr 8	^{14}C-Udr 4	^{3}H-Leu 1	^{14}C-Leu 1	^{75}Se-M 6	^{99m}Tc[4] 24	^{51}Cr 50	^{111}In 2	^{3}H-Tdr[5] 7	^{125}I-Udr 10
p. N.'s	0.5gm		8.81 ± 28	9.89 ± 44	7.96	7.96	6.29 ± 30	3.99 ± 60	30.7 ± 42 (24)[6]	30.8 ± 3	30.6 ± 22	1.93 ± 20 (5)
leen	0.4gm		7.53 ± 35	11.1 ± 29	4.92	6.78	11.4 ± 31	8.12 ± 50	18.0 ± 27 (51)	31.5 ± 8	14.1 ± 11	2.71 ± 20 (5)
t[7]	5.0gm		2.99 ± 33	2.74 ± 20	7.04	1.94	5.31 ± 25	-[8]	2.42 ± 45 (30)	3.60 ± 50	3.17 ± 75	25.0 ± 27
ne rrow	10.0gm		-	-	-	-	1.95 ± 22	-	5.17 ± 46 (47)	3.8 ± 2	-	4.3 ± 70
ymus	0.3gm		.082 ± 32	.112 ± 30	.19	.09	.12 ± 30	.01 (1)	.07 ± 75	.07 ± 20	.05 ± 55	.09 ± 85
D.L.s	Total output		4.9 (1)	7.2 ± 17 (2)	5.3 ± 6 (2)	8.4	11.3 (1)	.96 ± 80 (8)	16.1 ± 22 (6)	32.8 ± 20	15.0 (1)	

TABLE 3.5b

BODY FLUIDS

TISSUE	W.F.	ISOTOPE ^{3}H-Udr	^{14}C-Udr	^{3}H-Leu	^{14}C-Leu	^{75}Se-M	^{99m}Tc	^{51}Cr	^{111}In	^{3}H-Tdr	^{125}I-Udr
BLOOD	15.0ml	4.33 ± 27 (5)	1.75 ± 20 (2)	5.55	3.93	9.45 ± 30	5.51 ± 60 (21)	3.62 ± 50	5.4 ± 10	14.3 ± 85 (3)	1.76 ± 23
PLASMA	9.0ml	4.32 ± 92 (5)	.39 ± 26 (4)	5.94	1.98	8.17 ± 35	.76 ±120 (22)	.33 ± 25 (44)	.63 ± 15	2.7 ± 24 (3)	1.09 ± 33
BLOOD LEUKOCYTES[9]	15.0ml	-	1.83 (1)	-	-	-	-	1.88 ± 50 (39)	4.20 ± 10	-	.28 ± 25
WHOLE PERFUSATE	250ml	16.2 ± 13 (2)	2.72 ± 29 (2)	-	-	4.03 ± 15	-	1.18 ± 40 (41)	2.5 ± 4	-	3.18 ± 25
SUPERNATANT PERFUSATE	250ml	25.6 ± 33 (6)	1.45 ± 33 (4)	35.0	2.66	3.11 ± 30	-	.35 ±200 (47)	.25 ± 50	9.4 (1)	2.68 ± 40
CELLS IN PERFUSATE	250ml	2.54 ± 83 (2)	1.19 ± 42 (2)	.53	.67	.92 ± 60	-	1.28 ±110	2.0 ± 7	-	.25 ± 10
URINE 0-24 hrs	TOTAL[11]	3.0 (3)	3.0 (1)	-	.25	.06 (1)	23.8 ± 26 (8)	8.7 ± 20 (5)	.5 ± 10	3.5 (1)	-

TABLE 3.5c

NON-LYMPHOID TISSUES

TISSUE	W.F.	ISOTOPE	^{3}H-Udr	^{14}C-Udr	^{3}H-Leu	^{14}C-Leu	^{75}Se-M	^{99m}Tc	^{51}Cr	^{111}In	^{3}H-Tdr	^{125}I-Udr
OVARY	.1gm		-	-	-	-	-	-	.009 ± 50 (22)	.02 ± 20	-	.018 ± 31 (5)
UTERUS	.4gm		-	-	-	-	-	-	.008 ± 35 (23)	.02 ± 7	-	.064 ± 33 (5)
TESTIS	2.5gm		.54 ± 49 (7)	.09 ± 9	.60	.27	.69 ± 27	-	.026 ± 30 (25)	-	.20 ± 50	.092 ± 70 (5)
EPIDIDYMIS	3.5gm		.22 ± 12 (8)	.11 ± 23	.54	.18	.28 ± 40	-	.051 ± 45 (25)	-	.17 ± 78	.020 ± 80 (5)
SALIVARY GLAND	.3gm		.08 ± 41	.08 ± 54	.11	.04	.09 ± 50 (4)	-	.012 ± 95 (47)	.012 ± 25	.04 ± 128	.04 ± 170
BRAIN	1.5gm		.16 ± 99	.04 ± 86	.22	.21	.18 ± 30	-	.004 ± 100	-	.05 ± 159	.018 ± 90 (9)

TABLE 3.5d NON-LYMPHOID TISSUES

TISSUE	W.F.	ISOTOPE	^{3}H-Udr	^{14}C-Udr	^{3}H-Leu	^{14}C-Leu	^{75}Se-M	^{99m}Tc	^{51}Cr	^{111}In	^{3}H-Tdr	^{125}I-Udr
LIVER	10gm		3.9 ± 29	5.7 ± 10	21.6	5.6	7.0 ± 45	18.9 ± 90	11.4 ± 33	9.6 ± 25	2.1 ± 36	3.4 ± 50
KIDNEY	1.5gm		.59 ± 23	.46 ± 17	.72	.44	1.48 ± 45	6.69 ± 30	.58 ± 33	.60 ± 7	.26 ± 25	.18 ± 55
LUNG	1.2gm		1.41 ± 41	1.69 ± 32	1.47	2.22	2.01 ± 70	.34 (1)	2.81 ± 30 (49)	2.6 ± 20	4.02 ± 58	1.31 ± 50
SKIN	30gm		2.79 ± 68	1.31 ± 21	5.37	4.29	3.69 ± 50	2.64 ±150	.38 ± 33 (46)	.12 ± 10	2.48 ± 64	2.31 ± 45
MUSCLE	100gm		13.2 ± 44	5.33 ± 62	17.1	7.5	9.8 ± 30	2.6 ± 60	.85 ± 60 (43)	2.2 ± 10	10.9 ± 58	2.16 ± 50
TOTAL RECOVERY		10	66	40	95	41	59	64	73	83	79	47

Comparison of the values for each tissue shows wide variations between the results in various compartments with the different isotopes. The standard deviation about the mean for each compartment was of the order of 50% when experiments were pooled. The variation was consistently less between triplicate recipients within an experiment with a SD of 10-30% suggesting that the reproducibility is more dependent upon individual populations of cells rather than recipient variation.

The differences reflect the behaviour of the particular isotopic molecules after incorporation into the cell and also more importantly on subsequent release from labelled cells. The major differences will be delineated and their significance discussed with particular reference to the likelihood of a particular isotope producing results that reflect the labelled cell content of any compartment in the body.

None of the markers showed total accountability by 24 hrs after i.v transfusion of labelled cells. Even though most tissues were sampled minor variations would be expected as the precise total weight of each tissue was impossible to assay in each recipient. However, β emitters produced low total recovery when compared to γ emitters and this may be partly due to the more complex counting procedures used. ^{51}Cr produced consistent recovery and although higher than most other labels it was not so high as that found with ^{111}In. ^{14}C isotopes gave the lowest total recovery.

One major point of interest was the discrepancy between the % injected activity of ^{3}H as opposed to ^{14}C in tissues when both isotopes had been offered to the cells on the same carrier molecule - eg Uridine or Leucine. There was an excess of ^{14}C in lymphoid tissues but the converse was true in non-lymphoid tissues and cell free fluids. This result was confirmed in an experiment in which TDL were double labelled with ^{3}H and ^{14}C Udr and transfused into duplicate recipients. The activities in the various

compartments at 24 hrs after injection were expressed as the ratio of % injected ^{3}H to the % injected ^{14}C such that a ratio of less than one indicated an excess of ^{14}C and vice versa (Table 3.6).

For tritium isotopes with either carrier molecule there was significant activity in the supernatant of the perfusate fluid which was consistently in excess of the total amount estimated in the blood. This was also reflected in the ability of perfusion to reduce non-lymphoid tissue concentrations by a factor of more than two, which was not seen with eg ^{51}Cr.

In lymphoid organs such as spleen and lymph nodes ^{51}Cr, ^{111}In, and ^{3}H Tdr gave comparable high concentrations whilst ^{51}Cr also gave the lowest concentrations in non-lymphoid tissues usually by an order of magnitude even when compared to ^{3}H Tdr. ^{111}In compared very closely to ^{51}Cr in both lymphoid and non-lymphoid tissues particularly when it was certain that no ^{111}In-labelled protein was injected with the cells which could contaminate the tissues.

Two further approaches can be made to assess whether the label distribution reflects the behaviour of viable cells. If the labelled cells are functioning normally they will be able to migrate into central lymph via lymph nodes and in recipients with thoracic duct fistula will emerge with the lymph. The proportions of the input activity collected either associated with the emerging thoracic duct cells or in the supernatant lymph provides an assay of the behaviour of the labelled cells and gives some indication of the degree and fate of eluted label. Secondly, comparison of label distribution after injection of viable labelled cells with that of heat-killed cells revealed differences such that the viability of a test population could be assumed at least in part.

TABLE 3.6

Ratio of % inj. ^{3}H/% inj. ^{14}C in tissues 24 hrs after injection of thoracic duct lymphocytes double labelled with ^{3}H + ^{14}C Uridine.

Tissue	Recip.A	Recip.B
Injected cells	1.0	1.0
Spleen	.82	.80
Lymph node	.93	.88
Small intestine	.89	.86
Thymus	.73	.73
Liver	.94	.95
Lung	.85	.82
Kidney	1.20	1.29
Testis	4.31	5.89
Epididymis	1.81	2.90
Muscle	2.32	2.17
Brain	4.61	8.19
Skin	1.62	2.06
Plasma	4.7	4.7
Supernatant perfusate	18.6	19.3

When comparing the various isotopes the proportion of injected label bound to cells in the recirculating pool sampled at the thoracic duct again confirmed the differences seen in lymph nodes. ^{111}In gave the highest total recoveries over 24 and 48 hrs and at the other extreme 99mTc labelled cells virtually failed to appear in lymph. In this experimental model if double-labelled cells or mixtures of alternatively labelled cells are injected into the same recipient the appearance of cell-bound and free label can be measured and directly compared. The time course of cell associated label is well established with a peak of ^{3}H Udr labelled cells occurring at 18 hrs after injection. (Ford & Simmonds 1972)remembering that this compound is preferentially incorporated by T cells.

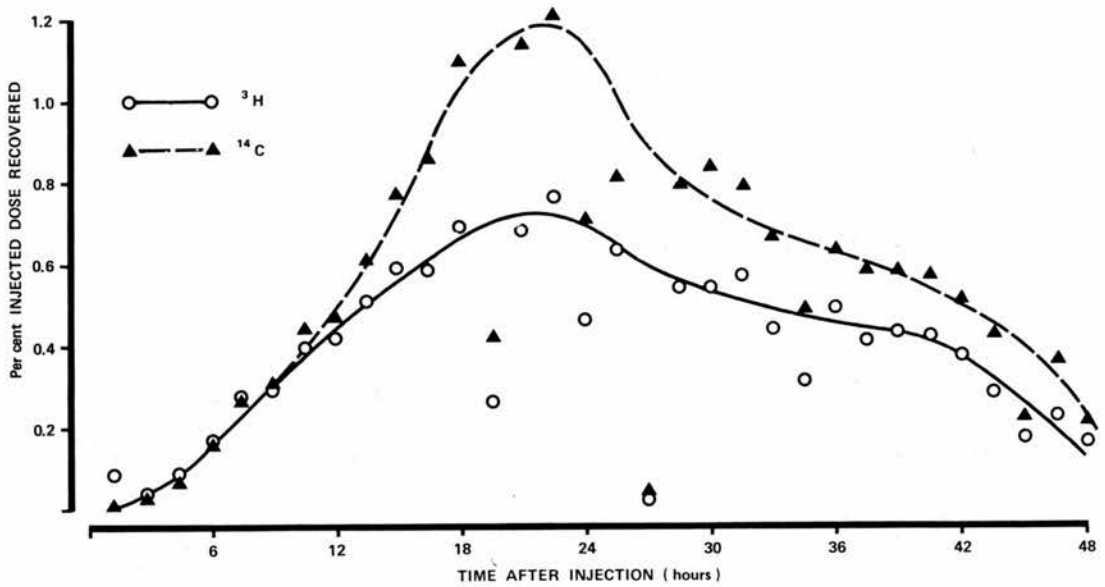
Figs 3.1 (a) and (b) show the results of an experiment in which TDLs double-labelled with ^{3}H and ^{14}C both followed the normal pattern but again more ^{14}C was measured than ^{3}H with the ratio of $^{3}H/^{14}C$ initially falling, but then reaching a plateau after several hours. In the supernatant lymph substantial ^{3}H was found soon after cell injection before any activity was found in the cell pellet arguing against the elution of this label from cells after collection into the flask as being the major source of this cell free label. Much less cell-free ^{14}C was found.

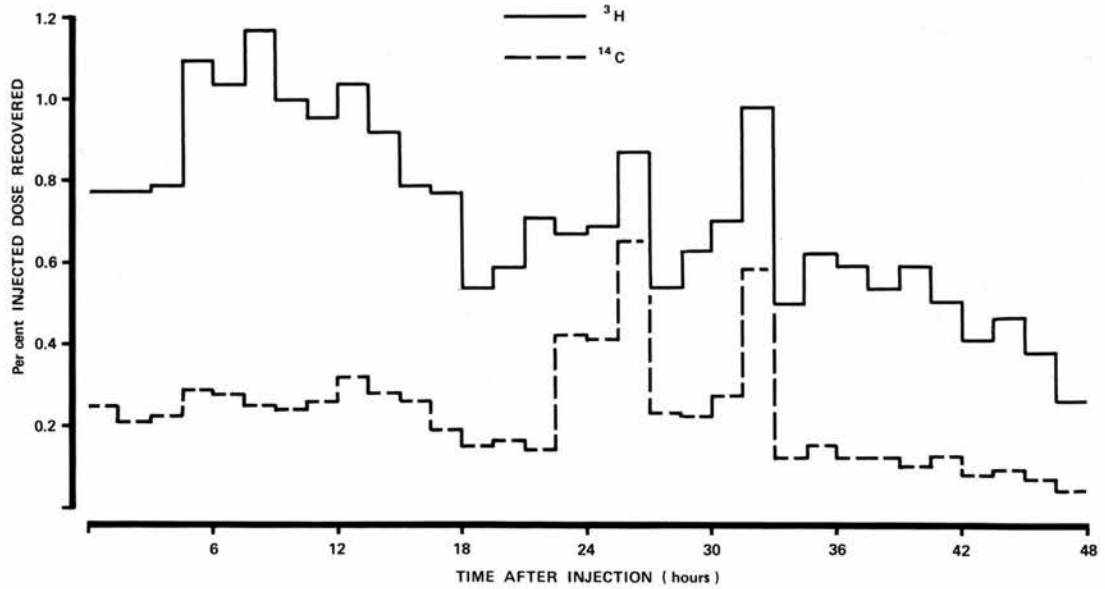
FIG 3.1

Recovery of ^{3}H and ^{14}C in the cells and supernatant lymph in 90 min sequential collections of lymph draining from a thoracic duct fistula of a rat injected at time zero with syngeneic TDL double-labelled with ^{3}H-Udr and ^{14}C-Udr.

Upper graph - percentage injected activity associated with the cell pellet.

Lower graph - percentage injected activity associated with the cell free lymph plasma.


Per cent INJECTED DOSE RECOVERED
1.2
1.0
0.8
0.6
0.4
0.2
^{3}H
^{14}C
6
12
18
24
30
36
42
48
TIME AFTER INJECTION (hours)


Per cent INJECTED DOSE RECOVERED
1.2
1.0
0.8
0.6
0.4
0.2
^{3}H
^{14}C
6
12
18
24
30
36
42
48
TIME AFTER INJECTION (hours)

In a similar experiment using a mixture of ^{51}Cr and ^{111}In labelled TDL and collecting sequential 45 min fractions, progressively more ^{111}In was recovered than ^{51}Cr, but again both curves followed the normal time course. Very little of either isotope was found in cell free lymph. However, the total recovery of ^{51}Cr was greater than that with ^{14}C-Udr (Fig 3.2 a & b).

An indirect estimate of elution of label from cells in vivo can be made by measuring urinary excretion of isotope. A substantial proportion of ^{99m}Tc was found in urine collected over 24 hrs - at least 25%. Significant quantities of ^{51}Cr - approx. 10% - were also found but much less of the other isotopes. This does not mean that label did not elute from cells but rather that such eluted label was not excreted in urine. For example, the output of ^{3}H in urine was approximately 3% in restrained but intact recipients. However, if such recipients also received an intravenous infusion of saline at 2.0ml/hr then the urinary output was sometimes over 30%. This agrees with results published by Goldschneider & McGregor (1968b) who found up to 50% of injected ^{3}H in either cell free lymph or urine up to 48 hrs after labelled cell transfusion.

The fate of eluted label may be partly discerned by examination of the tissue distribution of label after heat-killed labelled cell transfusion. Part of this distribution will follow the fate of phagocytosed cells and debris but the proportion found in cell free fluids may indicate how such released label may behave after viable cell transfusion. However, no direct evidence can be adduced to decide whether the release of label after such viable cell injection follows death of a minor proportion of the cells or continual gradual release of isotope from all labelled cells.

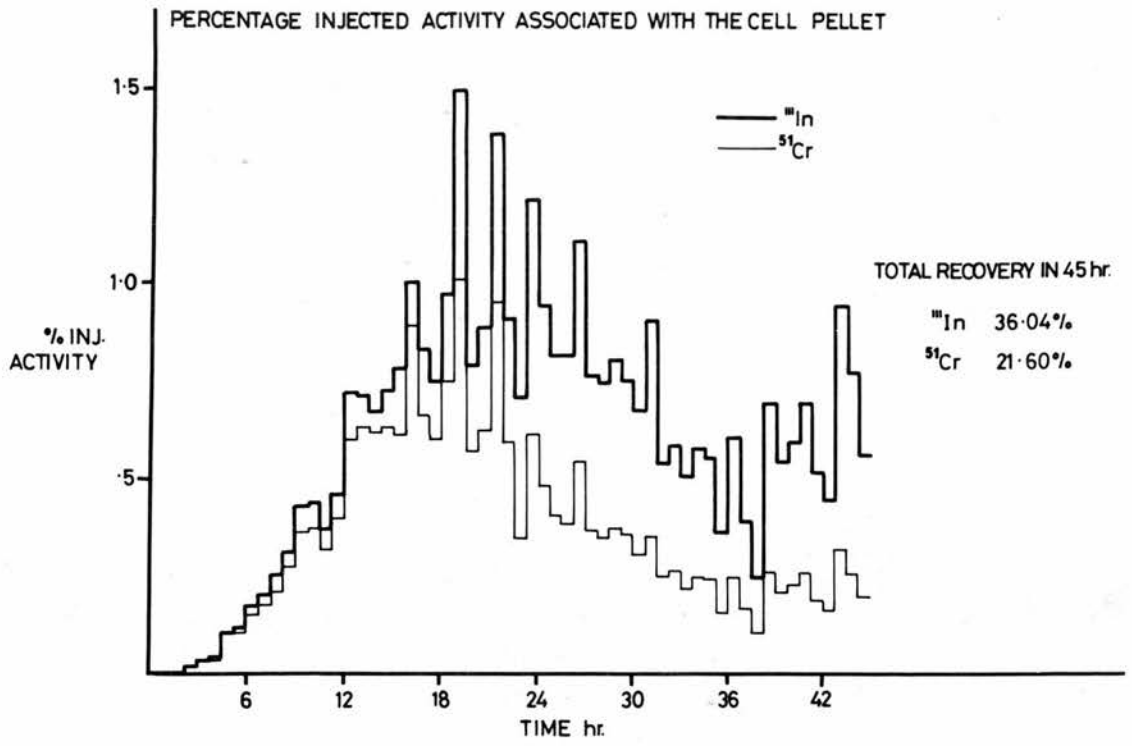
Table 3.7 shows the results of two experiments comparing the distribution of activity after injection of live and

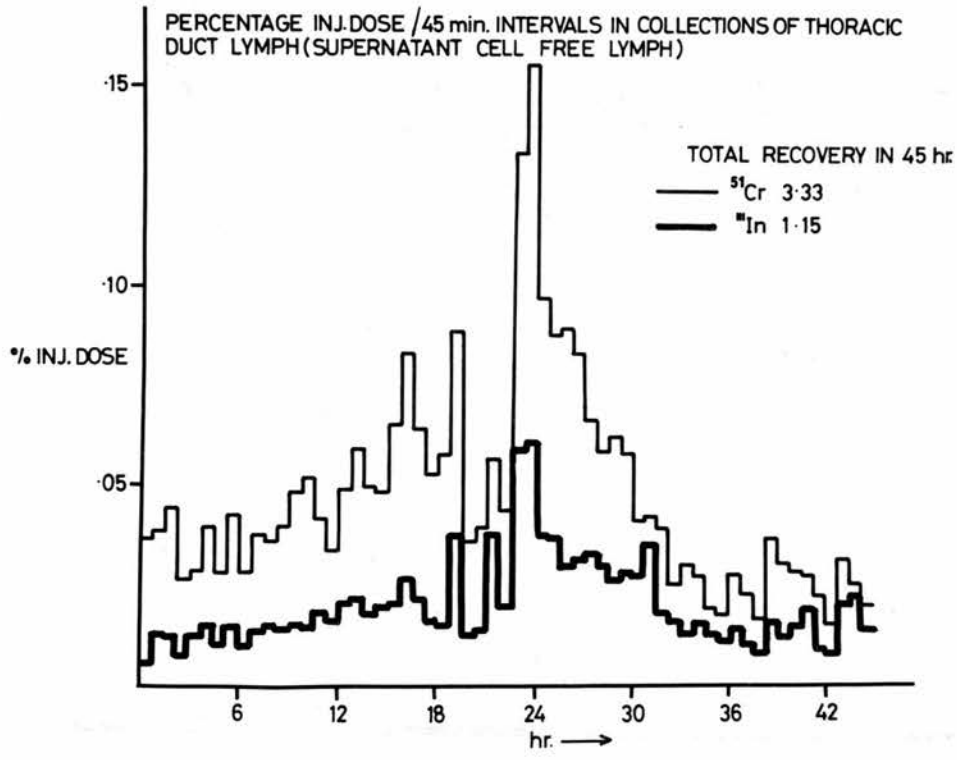
FIG 3.2

Recovery of ^{51}Cr and ^{111}In in the cells and supernatant lymph in 45 min sequential collections of lymph draining from a thoracic duct fistula of a rat injected at time zero with syngeneic TDL - half labelled with ^{51}Cr and the other half with ^{111}In-oxine.

Upper graph - percentage injected activity associated with the cell pellet.

Lower graph - percentage injected activity associated with the cell free lymph plasma.


PERCENTAGE INJECTED ACTIVITY ASSOCIATED WITH THE CELL PELLET
1·5
1·0
·5
% INJ. ACTIVITY
^{111}In
^{51}Cr
TOTAL RECOVERY IN 45 hr.
^{111}In 36·04%
^{51}Cr 21·60%
6
12
18
24
30
36
42
TIME hr.


PERCENTAGE INJ. DOSE /45 min. INTERVALS IN COLLECTIONS OF THORACIC DUCT LYMPH (SUPERNATANT CELL FREE LYMPH)
·15
·10
·05
% INJ. DOSE
TOTAL RECOVERY IN 45 hr.
^{51}Cr 3·33
^{111}In 1·15
6
12
18
24
30
36
42
hr. ⟶

heat-killed cells labelled with ^{51}Cr or ^{111}In expressed as % injected dose/organ.

The major proportion of heat-killed cell-associated label localized rapidly in the liver and this high concentration was maintained and possibly increased by 24 hrs. A significant proportion of either label was present in the plasma by 1 hr and these concentrations fell by 24 hrs though for apparently different reasons. ^{51}Cr was excreted in the urine. ^{111}In was not excreted but rather continued to accumulate in liver, spleen and bone marrow probably due to presence of transferrin to which ^{111}In binds avidly (Thakur 1977) as well as the accumulation of labelled cell debris in the reticulo-endothelial system. Concentrations of ^{111}In in non-lymphoid tissues were in some cases greater after dead cell injection than after viable cell injection, but no meaningful correlation between tissue levels and cell-free plasma levels occurred in either case.

The avid binding of free ^{111}In to protein and its distribution may account for the somewhat analogous results in the experiment of Table 3.8. Cells were labelled with ^{111}In in medium which contained a trace of protein and from which the cells were washed only once. Recipients of these cells together with ^{51}Cr-labelled TDL showed the tissue concentrations listed in Table 3.8. The lymphoid organs had an apparent deficit of ^{111}In labelled cells compared to ^{51}Cr in contrast to the experiment detailed in Table 3.7. However, the concentration in lymph nodes and spleen did alter with time in the normal way and the discrepancy appeared to be due to the high plasma and liver concentrations evident soon after injection ($\frac{1}{2}$ hr). The bone marrow showed an early deficit and a late excess of ^{111}In that is consistent with a deficit of labelled cells and an excess of labelled protein the latter of which could account for the continued increase after 20 hrs. The other interpretation would be that[a] substantial proportion of the initial population of ^{111}In but not ^{51}Cr labelled cells were dead at the time of injection.

TABLE 3.7

TISSUE DISTRIBUTION OF RADIOACTIVITY 1 HR AND 24 HR AFTER I.V. INJECTION OF EITHER (^{51}Cr) OR (^{111}In) LABELLED TDLs IN DUPLICATE SYNGENEIC WEIGHT MATCHED RECIPIENTS PERFUSED AT TIME OF DEATH.

MEAN % INJ. ACTIVITY/WHOLE ORGAN OR VOL OF BODY FLUID

(N.B. Great care was taken to ensure that (^{111}In) labelling was carried out in protein free medium with thorough washing of the cells)

Upper value for each tissue - Viable cells
Lower " " " " - Heat-killed cells

TISSUES	ONE HOUR		24 HOURS	
	^{51}Cr	^{111}In	^{51}Cr	^{111}In
LYMPH NODES	3.4	2.4	26.5	30.8
	.06	.12	.11	.26
SPLEEN	47.7	56.2	22.0	31.5
	1.7	2.6	2.6	5.2
LIVER	11.3	10.8	9.0	9.4
	37.1	39.9	38.1	53.5
KIDNEY	.28	.39	.29	.30
	4.8	2.0	5.6	1.8
GUT	4.5	1.9	2.9	3.6
	.52	.68	.27	.75
LUNG	4.5	4.3	1.8	1.6
	22.7	23.1	1.5	2.2
BONE MARROW	14.7	18.1	2.3	2.1
	3.9	3.2	7.2	10.1
OVARIES	.11	.01	.05	.02
	.04	.06	.03	.04
UTERUS	.04	.01	.04	.02
	.05	.12	.07	.16
THYMUS	.06	.11	.21	.07
	.03	.04	.02	.04
SALIVARY GLAND	.02	.01	.06	.01
	.03	1.2	.04	.62
SKIN	.45	.47	.28	.58
	2.0	2.9	1.5	3.4
MUSCLE	1.37	1.23	.56	1.12
	4.7	6.1	5.0	6.1

TABLE 3.7 (Contd.)

FLUIDS	ONE HOUR		24 HOURS	
	^{51}Cr	^{111}In	^{51}Cr	^{111}In
WHOLE BLOOD	3.7	3.2	3.0	2.8
	5.8	6.8	1.2	2.3
PLASMA	.27	.44	.18	.35
	4.9	5.7	1.1	1.3
BLOOD LEUCOCYTES	1.3	1.5	1.3	2.2
	.4	1.0	.2	1.1
WHOLE PERFUSATE	2.2	3.2	1.6	1.4
	4.9	5.9	1.3	3.6
SUPERNATANT PERF.	.6	.5	.2	.2
	3.3	3.8	1.0	2.5
CELLS IN PERFUSATE	1.5	2.5	1.3	1.2
	1.0	1.6	.4	2.1
TOTAL RECOVERY (viable cells)	88.0	96.1	66.3	82.1

TABLE 3.8 TISSUE DISTRIBUTION OF (^{51}Cr) AND (^{111}In) AFTER INJECTION OF A MIXTURE OF LABELLED CELLS AFTER ½, 2, 20 AND 45 HOURS. TRIPLICATE SYNGENEIC RECIPIENTS.

(N.B. (^{111}In) labelling medium contained a trace of protein and these cells were only washed once)

MEAN % INJ DOSE /GM TISSUE OR /ML FLUID

TISSUES	0.5HR		2HR		20HR		45HR	
	^{51}Cr	^{111}In	^{51}Cr	^{111}In	^{51}Cr	^{111}In	^{51}Cr	^{111}In
LYMPH NODES	1.4	.66	2.0	.86	33.2	10.7	28.1	9.9
SPLEEN	76.8	23.2	93.8	27.2	54.2	20.1	36.6	15.3
LIVER	2.6	3.9	2.2	4.1	1.8	4.6	1.9	4.5
KIDNEY	1.1	1.3	1.0	1.2	1.1	1.9	1.2	1.7
GUT	.67	.21	.88	.26	1.1	.54	.63	.32
LUNG	18.5	15.1	3.0	2.9	2.2	1.8	1.4	1.1
BONE MARROW	1.8	.64	2.5	1.0	.51	.62	.61	.69
OVARIES	.25	.42	.19	.44	.10	.48	.15	.47
UTERUS	.06	.26	.05	.33	.07	.45	.06	.46
THYMUS	.06	.07	.05	.06	.14	.19	.13	.19
SKIN	.05	.09	.03	.22	.03	.22	.03	.20
MUSCLE	.03	.05	.03	.06	.02	.09	.02	.07
BLOOD	.76	1.9	.29	1.2	.31	.43	.28	.14
PLASMA	.26	2.5	.21	1.9	.09	.49	.04	.09
BLOOD LEUCOCYTES	.38	.16	.07	.07	.15	.28	.13	.05
PERFUSATE	.029	.054	.017	.050	.016	.019	.012	.001
SUPERNATANT PERF.	.012	.057	.008	.049	.005	.013	.001	.003
CELLS IN PERF.	.024	.010	.010	.004	.008	.004	.005	.004

S.E. consistantly of the order of 10% of mean values excluded for the sake of clarity.

Tissue concentrations at 24 hrs after injection of ^{75}Se labelled cells showed values in lymphoid organs and TDL intermediate between ^{51}Cr and ^{14}C but high concentrations were found in non-lymphoid tissues and plasma. There was no excess of ^{75}Se in the perfusate over the blood levels and the radioactivity in plasma was associated with 5% TCA precipitated macromolecules, confirming the observations of Rose & Micklem (1976) in the mouse.

^{125}Udr is metabolically incorporated into DNA and therefore labels only those cells in S-phase. In lymph the minority population of lymphoblasts therefore are the only cells labelled, and since Gowans & Knight (1964) and Hall et al (1972) showed that these cells behave differently from the majority population of small lymphocytes in that they (i) preferentially migrate to the small intestinal lamina propria, (ii) are found in much lower proportions in secondary lymphoid organs, and (iii) migrate poorly into thoracic duct lymph, direct comparison of isotope behaviour with labels in different cell populations can only be made as far as the behaviour of the cell-free portion of eluted label is concerned. The majority of ^{125}I in the blood was in the plasma and there was also some excess activity dialysed out by perfusion suggesting some degree of widespread redistribution of cell-free label contaminating tissues similar to that seen with tritium. The pattern of tissue distribution confirmed published results suggesting that a major proportion of the labelled cells were viable.

Distribution of ^{99m}Tc in non-perfused recipients showed that a high proportion of the label was accounted for in the liver, kidneys and urine. Very variable low concentrations were found in lymph nodes and little or no activity was found associated with cells in draining thoracic duct lymph. All these factors are suggestive of damage to the cells.

^{99m}Tc and ^{111}In are both isotopes with high outputs of

gamma photons that are efficiently detectable by external imaging devices and as such provide potential labels for the study of cell migration in vivo with non-invasive techniques. Fig 3.3 and Fig 3.4 show respectively images obtained with a gamma camera 18 hrs after the intravenous transfer of TDL labelled with approximately 300μCi of isotope.

Fig 3.3 shows the distribution after transfer of 1320 x 10^6 TDL labelled with 320μCi ^{99m}Tc. There is marked concentration in liver, spleen, kidneys and bladder and the cervical lymph nodes are just discernable.

Fig 3.4 shows the distribution pattern of activity 18 hrs after iv injection of 200 x 10^6 TDL labelled with 300uCi ^{111}In-oxine. The cervical lymph nodes are easily discerned and several other nodes in thorax and abdomen as well as the prominent spleen and liver can be seen. Fig 3.5 shows the cervical region of the same animal viewed with a pinhole collimator thus giving a greater degree of magnification.

Fig 3.3

Gamma camera image of ventral aspect of an anaesthetized rat injected iv 18 hrs previously with syngeneic TDL labelled with 320μCi ^{99m}Tc. 15 min exposure using a multihole collimator.

The liver, spleen and kidneys are active as is the bladder (arrowed). The cervical lymph nodes appear as a faint area of radioactivity at the upper part of the image.

Mag. x 1.

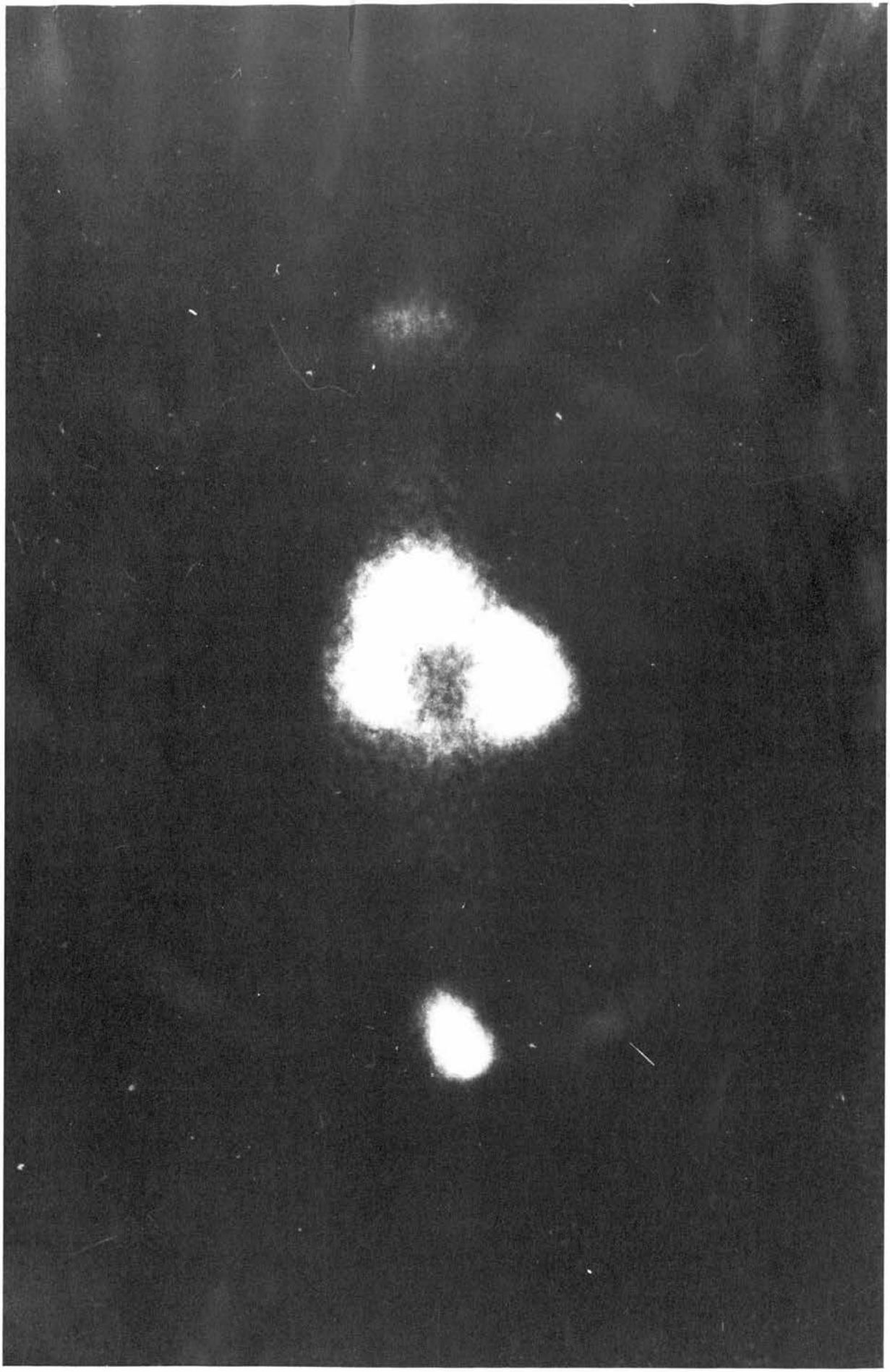


FIG 3.4

Gamma camera image of ventral aspect of an anaesthetized rat injected iv 18 hrs with 200×10^6 syngeneic TDL labelled with 300μCi ^{111}In-oxine 15 min exposing using a multihole collimator.

Liver and spleen are very active but many lymph nodes are also obvious particularly the cervical group.

Mag. x 1.5

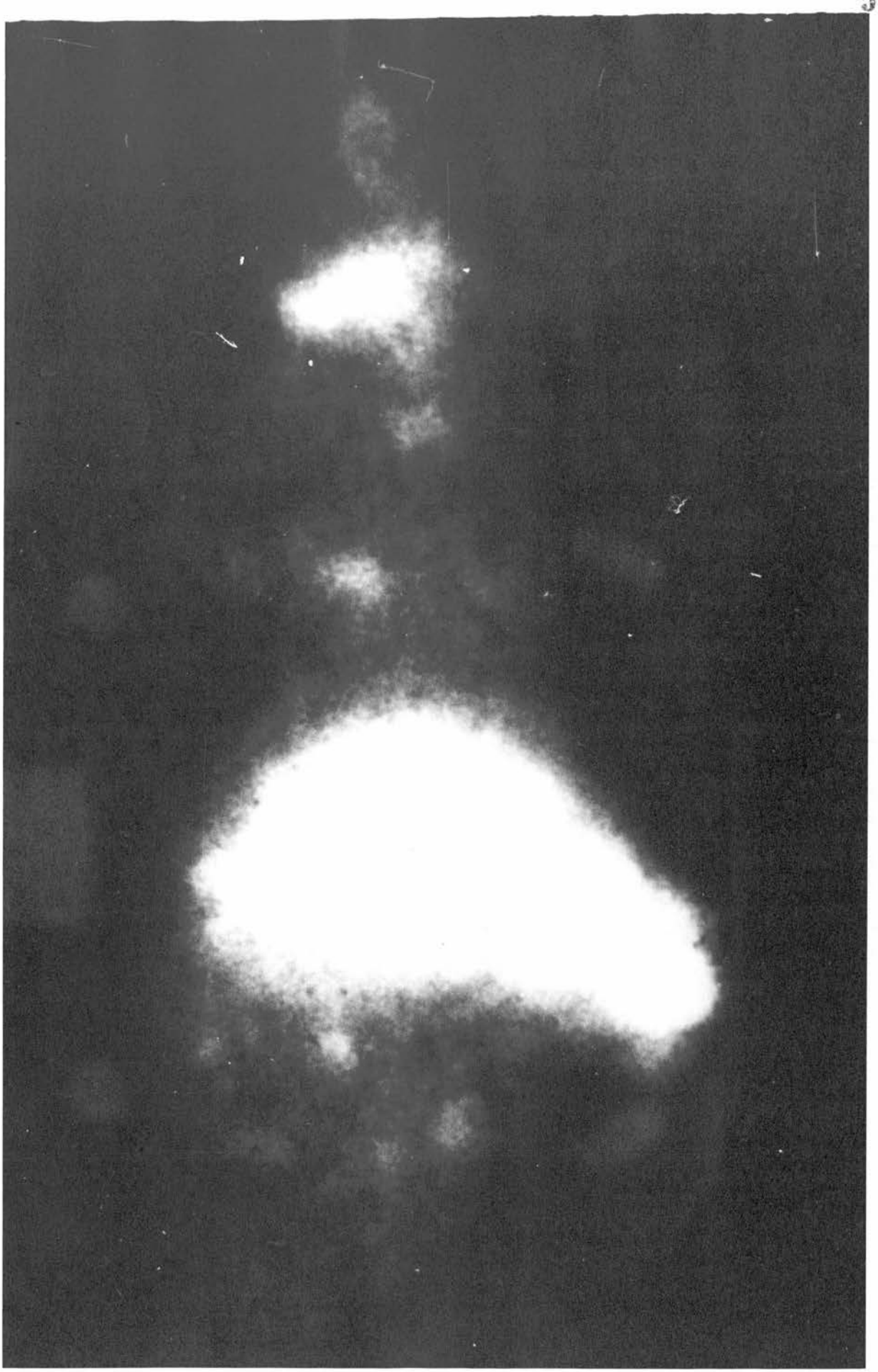
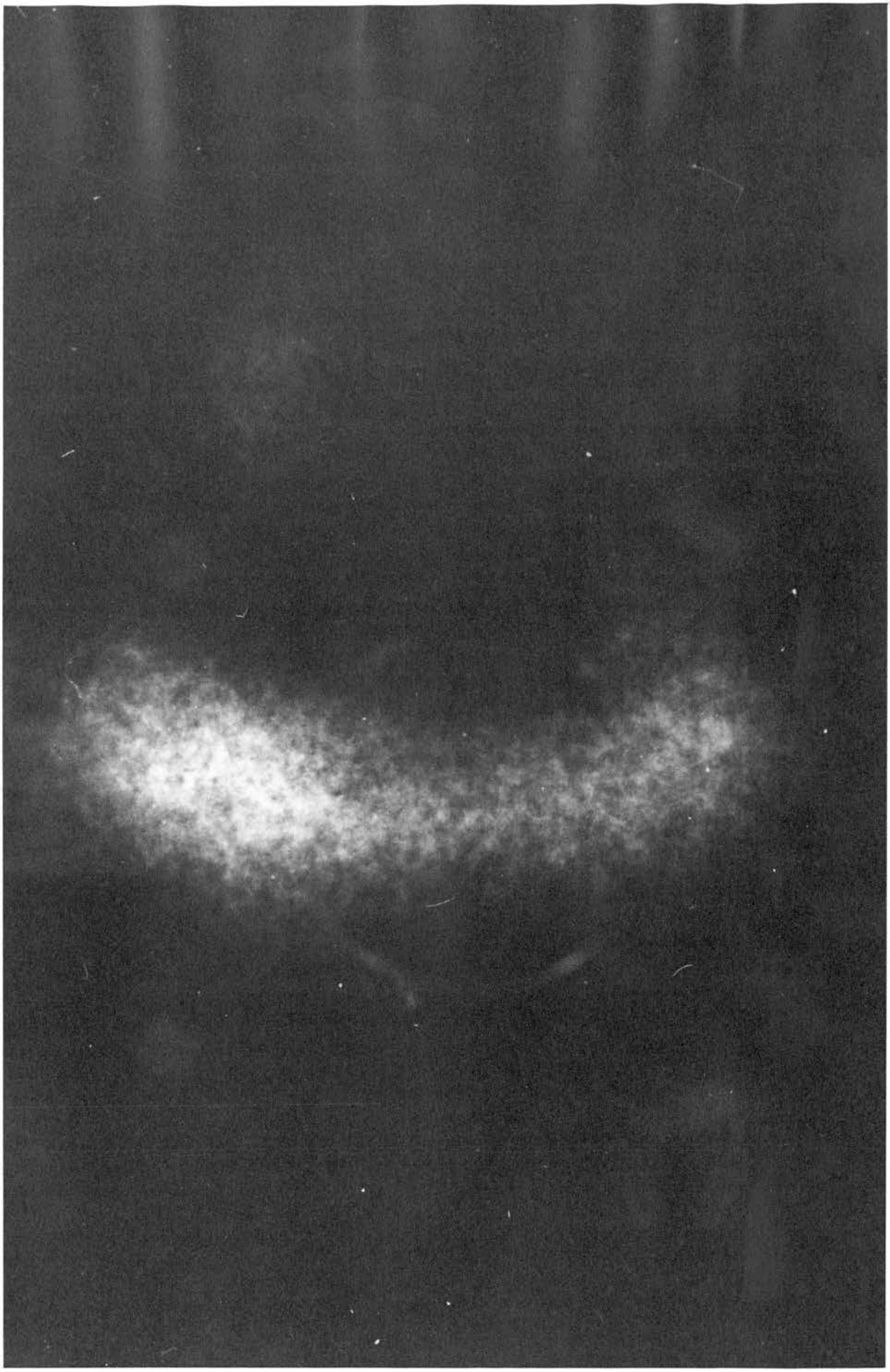


FIG 3.5

Same animal as Fig 3.4.

Gamma camera image using a pin-hole collimator.
15 min exposure over cervical region.
Areas of activity are individual lymph nodes weighing approximately 1.0g.

Mag. 15



DISCUSSION

Several features of the behaviour of radioactive marker molecules following their uptake by thoracic duct lymphocytes are apparent from the results.

When a radioactive molecule passes across the cell membrane it may be incorporated into the cell structure in a variety of ways or it may remain in solution in the cytoplasm such that it may leave the cell again unchanged at some later time. If the latter occurs then the molecule may be available for uptake and incorporation by another cell and in vivo by other cell types. This true re-utilization is one potential source of artefactual data when total tissue activity is equated with introduced labelled cell content.

Any molecule that is truly incorporated into intracellular structures shares the fate of the particular macromolecule to which it is attached and may therefore have a variable biological half-life within the cell depending upon the metabolic pathways involved. For example ^{3}H Tdr or ^{125}IUdr are incorporated into DNA, a relatively stable molecule. On the other hand uridine is a precursor of RNA which may have a great variety of lifespans as will amino-acids synthesized into proteins. The catabolism of the macromolecules may allow release of the isotopic atom from the cell in a new molecule which will determine its ultimate fate.

The same principles hold true for the Category (iii) non-

metabolic labels which after uptake bind by non-specific chemical forces rather than by biosynthetic incorporation. However, once bound, the fate of the marked atom will then follow that of the molecule to which it is bound depending in part upon the relative binding activities of the molecules present in the immediate environment. Whether re-utilization will take place on release from labelled cells will again depend upon the type of molecule to which it is bound or if truly free the chemical state of the ion. For example it has been reported that ^{51}Cr on release from cells is bound to molecules with characteristics of small peptides or amino acids (Sanderson 1976, Ronai 1969).

The suitability of an isotope for tracing cells to areas of low cell concentration by the indirect scoring method of measuring total tissue activity depends upon the inter-relationship of several factors:

a. the stability of the attachment of the label to the cells.

b the rate of elution of label from the test cells.

c the rate of uptake of eluted label by other cells in the test tissue.

d the appearance of label in tissue fluids.

e the rate of removal of the label from the body by excretion.

f the viability and normal behaviour of the labelled cells.

In the ideal situation (a) and (f) would be maximal and the remaining factors would not interfere. Given that this ideal situation does not exist the relative advantages and disadvantages of each marker will be considered.

A ^{3}H Thymidine in vivo

This nucleotide is incorporated into DNA and as such should be part of a stable structural molecule in long-lived non-dividing recirculating lymphocytes (both T and B cells). The cells were labelled in a physiological environment and were transferred to recipients with minimum manipulation. The results suggest normal migratory function of such lagelled cells by the high lymph node concentrations and substantial recirculation into lymph. However, elution and redistribution of ^{3}H does occur as shown by significant concentrations in plasma and in the cell free perfusate. This suggestion is confirmed by the significantly higher concentrations in non-lymphoid tissues of ^{3}H as opposed to ^{51}Cr.

Several mechanisms may be involved in the appearance of such cell free label:

(i) A proportion of the transferred labelled cells may die on transfer following in vitro manipulation or because they have reached the end of their normal life. Catabolism could then release ^{3}H from the DNA.

(ii) A proportion of the ^{3}H in transferred cells may not be incorporated into DNA but may rather be of low molecular weight and in dynamic equilibrium with the extracellular fluid which in the original labelled donor would still contain ^{3}H. On transfer net redistribution could occur with appearance of cell free ^{3}H in the extracellular fluid.

(iii) A proportion of the labelled DNA may have a metabolic turnover dissociated from that of the chromosomes as suggested by Harris et al (1973; 1976).

(iv) There may be atomic exchange of hydrogen atoms at the surface of molecules with a net elution of ^{3}H in the new non-radioactive environment.

In fact a standard method of tritiating compounds involves such an exchange in the presence of excess ^{3}H and a catalyst.

There is no evidence which would exclude any of these factors and all may operate.

Tritiated DNA is usually considered stable as measured by autoradiographic grain counts over samples of a cell population over long time periods (Theml et al 1973).

The present results raise the possibility that low molecular weight activity may be washed out during processing even of cell semears which would prevent the detection of <u>total</u> intracellular radioactivity.

The source of eluted tritium is less important in the present circumstances than its fate which appears to be widespread contaminatory redistribution. The advantages of this label for autoradiographic studies of cells of known age in areas of reasonably high frequency make the expense in terms of isotope and time worthwhile for certain studies, but the advantages of using a non-traumatized closely defined (by age) population are nullified when quantitating non-lymphoid tissue cell content by assaying total radioactivity as over-estimation will occur.

B <u>Uridine</u>

In the rat this precursor of DNA is more efficiently incorporated by T cells than B cells (Howard et al 1972b). This differential uptake must be taken into account when the activity associated with a mixed cell population such as TDL is being followed. In addition not all of the radioactive uridine that is absorbed during culture may be incorporated into RNA immediately as suggested by the finding of Gowans & Knight (1964) that an increase in macromolecular TCA resistant activity detected by grain counts over cells labelled with ^{3}H adenosine more than 27 hrs after iv transfusion as compared to the starting population

immediately after labelling. This low molecular weight activity, if not subsequently incorporated, may be released into the environment - ie the recipient animal.

Avoidance of such contaminatory eluted label might be attempted by assaying only RNA from tissues. This may not be as helpful as expected for the following reasons. Extraction of RNA is unlikely to be total and efficiency may vary between tissues - eg attempts to extract macromolecular radioactivity with TCA from homogenized renal tissue gave total yields of only 60% of that measured in raw digests even when the precipitate and supernatant fractions were combined. In addition the amounts of label incorporated into RNA within the cell may vary with time. Measurement of specific activity of RNA in various tissues is also open to the inherent variation in RNA concentration in different tissues.

The variation in total tissue concentrations between ^{3}H and ^{14}C when the same cells were double-labelled with these isotopes on the uridine molecule must reflect the fates of different parts of uridine molecule. Thus tritium eluted from labelled cells to a greater extent than ^{14}C as shown by the relative deficit of ^{3}H lymphoid tissues and thoracic duct lymph cells. The converse was true in areas of expected low cell concentration and cell free fluids. The excess ^{3}H released by perfusion with the corresponding fall in tissue activity is not seen with other labels. That this label is available for excretion in urine under conditions of high fluid intake from an iv saline infusion suggests that the tritium moves with the body water pool.

The elution from uridine could be due to those factors already suggested for ^{3}H Tdr but the observation that a similar pattern of elution still occurs (28% in perfusate and 11% in plasma) when labelled cells are double passaged through an intermediate recipient suggests that the elution is gradual and continuous and

cannot be accounted for my non-incorporation of low molecular weight uridine alone. The relative intracellular conservation of ^{14}C also argues against the non-incorporation idea as this should also affect the ^{14}C form of the molecule. This conservation of ^{14}C could occur if catabolic products of RNA separate the 5-H from the 2-C or if the tritium exchanges at the surface of the intact molecule. The low total accountability of ^{14}C however argues that ^{14}C does also elute although at a slower rate but the failure to detect such eluted ^{14}C is unexplained. One possibility is that the ^{14}C was excreted via the lungs as $^{14}CO_2$. In a not totally satisfactory experiment all expired CO_2 from a recipient of ^{14}C Udr labelled TDL was collected from a gas-tight box for 24 hrs by precipitation as Barium carbonate. The precipitate failed to show significant counts when it was dissolved with tetrasodium EDTA and added to a liquid scintillation cocktail.

The widespread redistribution of low molecular weight ^{3}H could provide an alternative explanation of the observed increases in TCA insoluble/TCA soluble ratio of radioactivity in lymphoid tissues with time after ^{3}H labelled cell injection when no increase in this ratio was measured in non-lymphoid tissues. (Goldschnieder & McGregor 1968b, Ellis & de Sousa 1974). These observations have been cited as evidence that lymphocytes continue to synthesize RNA from labelled precursors only in lymphoid tissues. However, such alterations in this ratio would also be produced by net elution and redistribution of TCA soluble label from areas of high cell content to all other areas including those of low cell content. Thus the TCA insoluble fraction of lymphoid tissue would increase with time because of cell accumulation and at the same time the eluted TCA soluble activity would show a net efflux and both factors would push the ratio in the same direction. However in non-lymphoid tissues containing few labelled cells and thus little TCA insoluble ^{3}H the net inflow

of TCA soluble ^{3}H would tend to stabilize or even reduce the ratio. The ability of lymphocytes to continue RNA synthesis from radioactive precursors is suggested by the increase in RCA resistant grain counts in ^{3}H adenosine labelled cells with time as already stated (Gowans & Knight 1964) but there is no evidence in favour of a restriction of this ability to lymphocytes within lymphoid tissues.

Thus uridine because of its rapid rate of elution and particularly because of contamination of tissues with cell free label is unlikely to give accurate estimates of labelled cell content using indirect methods. Nevertheless ^{14}C is likely to give more realistic results than ^{3}H.

C Leucine

This amino-acid does not show the differential uptake between T and B cells but otherwise shows the same range of problems experienced with Uridine - ie elution is rapid and particularly with tritium contamination is high.

D ^{75}Se-L Selenomethionine

The incorporation of this analogue of the sulphur containing amino acid methionine into lymphocytes without impairing their function was reported by Rose & Micklem (1976) and Bainbridge (1976). It has the potential advantage of emitting γ rays but the biological behaviour as shown by these results suggests that elution is substantial and this may occur unevenly between lymphoid organs as suggested by Bainbridge (1976). The eluted label binds to plasma proteins and may also be re-utilized by other tissues as shown by the higher non-lymphoid concentrations than ^{51}Cr and the failure of perfusion to dialyse out more activity than could be accounted for in the blood.

E Sodium ^{99m}Tc Pertechnetate

The work of Barth et al (1974) using murine lymphocytes suggested that high cell specific activities with stable labelling could be achieved with this label although such potentially toxic compounds as stannous chloride were used in the labelling method. Preliminary studies suggested this step was indeed excessively toxic and sodium chromate also used as an adjuvant for labelling did not improve the situation. A wide range of labelling conditions was investigated with great variability in the performance of labelled cells; these never produced high lymphoid tissue localization but consistently showed much activity in liver, kidneys and urine. The ability of such labelled cells to recirculate into thoracic duct lymph was severely impaired. Thus the potential advantages of achieving greater detectability in non-lymphoid tissues by having a high cell specific activity and of being able to visualize labelled cells with external scanning devices were not realized. A recent report of the distribution of ^{99m}Tc labelled peripheral blood lymphocytes in the rabbit measured by radioassay of dissected tissues or by external scanning with a gamma camera gave similar data to that shown here (Gobuty et al 1977). However these authors interpreted their results in terms of cell distribution which seems most unlikely.

F Sodium ^{51}Cr Chromate

This radionuclide has had widespread use in labelling a variety of cell types including TDL, after the original description of the method by Bainbridge et al (1966). Like other non-metabolically incorporated labels it is taken up by all cell types thus necessitating great purity of the starting population. Because of the low efficiency of uptake (2% of isotope added to the culture) low cell specific activities are achieved and this can only be safely counteracted by injecting more cells as increasing the labelling cell dose may introduce two

potentially toxic factors: (i) the concentration of chromate ion may be chemically toxic; (ii) radiotoxicity is likely to be dose dependant. Thus the ability of ^{51}Cr-chromosome marked murine lymphocytes to survive in vivo and proliferate on subsequent exposure to lectins was reduced compared to non-radiolabelled similarly chromosome marked cells (Doenhoff & Davies 1976). Also rat TDL labelled at 100μCi/ml showed significant impairment of activity in a local graft versus host assay whereas the same dose of cold chromate or labelling at 25μCi/ml showed no consistent impairment (B Rolstad personal communication). In one experiment TDL labelled at 10μCi/ml ^{51}Cr showed slight impairment of GVH activity in a similar rat popliteal node GVH assay.

On the other hand the ability to proliferate may be too stringent a test of normal function as far as migratory behaviour is concerned. The proportional distribution of the same population of TDL labelled at 10μCi, 30μCi, or 100μCi/ml showed no significant differences in either tissue distribution or draining thoracic duct lymph cells as shown in Table 3.9.

TABLE 3.9

Effect of Labelling Dose of ^{51}Cr on Tissue Distribution and Migration of Labelled Cells into Thoracic Duct Lymph after IV Injection

% Inj. Dose/gm Tissue or Total Fluid Vols (Perf at 24 hrs)

Tissues	10μCi/ml	30μCi/ml	90μCi/ml
Lymph node	41.4	26.9	35.1
Spleen	34.3	35.6	38.8
Liver	.89	.55	1.2
Kidney	.86	.60	.68
Bone marrow	.19	.19	.36
Thymus	.08	.03	.12
Skin	.01	.008	.008
Muscle	.008	.011	.008
Brain	.001	.001	.001
TDL cells (0-21 hrs)	9.7	10.0	8.6
TDL sup " "	.75	1.0	.95
Urine	7.4	9.1	12.8
Blood	6.2	4.8	5.3
Plasma	.23	.16	.27
Perfusate (whole)	2.6	1.8	1.4
Perfusate (cells)	3.2	1.5	1.2

However, a routine dose of 10μCi/ml was used throughout the remainder of the project.

The non-specific uptake into all cell types necessitated the removal of contaminating erythrocytes as these could amount to ten percent of the population of TDL without obvious bleeding or discolouration of the lymph. The activity in blood was not due to reutilization in vivo but to prelabelling. Autoradiographs of smears of ^{51}Cr labelled TDL showed labelled red cells as long as Haematoxylin and eosin rather than Unna-Pappenheim stains were used. Erythrocytes are not well stained with the latter preparation and are therefore easily overlooked. Centrifugation over Ficoll/Hypaque was found to be the most efficient method of removing this low level contamination.

Uptake of ^{51}Cr by cells is not uniform but tends to depend upon cell size. Large cells incorporate more isotope than small (Eyre et al 1970). As the larger cells in TDL are immunoblasts and behave differently to small lymphocytes, it is inherently difficult to apportion accurately the contribution of the sub-populations to the total concentration of radioactivity in any tissue at a given time, even if the proportion of the total label associated with this minority population was known precisely.

However, the results suggest that ^{51}Cr labelled cells are most likely to reflect the physiological distribution of cells in non-lymphoid tissues because activity was the lowest recorded of all the labels used. This is almost certainly due to the efficient excretion of any eluted label with little evidence of redistribution or reutilization. However some elution does occur, since ten percent of the injected activity was found in urine and also recovery in thoracic duct cells was lower than that of ^{111}In labelled cells (Fig 3.3). Elution was also seen in culture where by 3 hrs there was 30% spontaneous release of ^{51}Cr. In comparison only 5% of ^{111}In was released from TDL cultured at

5×10^6/ml in RPM1 + 10% FCS at 37°C in vitro.

These advantages outweigh the disadvantages of the non-uniform uptake but poor results in autoradiographs of tissue sections (probably due to loss of activity during processing) preclude the use of this technique to confirm directly how differences in total tissue ^{51}Cr activities reflect differences in cell content.

G ^{111}In-Oxine

The preliminary results of labelling lymphocytes with this compound confirmed the findings of Thakur et al (1976 a & b; 1977) when labelling other cell types (leukocytes and platelets) and suggest that this label may be extremely useful in both experimental and clinical investigations. The rapid uptake, high cell specific activity obtainable, and low rate of elution taken together with the normal migratory behaviour, establishes this method as a useful alternative to ^{51}Cr labelling for experimental situations with the added advantage of being more efficiently detected by external scanning devices. In addition the ability to label cells heavily should allow a greater degree of detectability in areas of low cell concentration particularly if the number of cells available for labelling is limited. A possible disadvantage of ^{111}In is the binding of this ion to transferrin on elution from dead or living cells. It is therefore not excreted and may contaminate some tissues.

With well washed labelled cells the concentrations of ^{111}In in non-lymphoid and lymphoid tissue were slightly higher than with ^{51}Cr, perhaps due to the greater elution of ^{51}Cr.

Assay of heavily labelled TDL in a local GVH assay did show an abolition of proliferative activity but this again may be too stringent a test of normal function for migration studies.

H ^{125}I-Iododeoxyuridine

As a label for DNA synthesizing lymphoblasts this analogue of Tdr has the advantages over tritiated Tdr of being a gamma emitter and therefore allowing examination of larger samples and also of having slightly better elution characteristics. However elution does occur and at least part of the free label is widely distributed as shown by whole body perfusion which yields more in the perfusate than can be accounted for by the blood. Also ^{125}I has a greater radiotoxicity in DNA as shown by reduced growth in labelled tumour cell cultures (Hofer & Hughes 1971) and by alterations in migration characteristics of murine "activated T.TDL" when increasing labelling doses of ^{125}Udr reduced splenic localization probably reflecting increasing cell damage (Sprent 1976a). Thus 1μCi/ml is an upper limit of acceptable in vitro labelling dose for 1 hr in culture.

Non-lymphoid tissue concentrations are thus likely to over estimate lymphoblast cell content but this artefact does not completely vitiate the use of this label to gain some insight into the migratory behaviour of such labelled cells in most sites.

CONCLUSIONS

The wide variations in tissue distribution of radioactivity measured after iv injection of TDL labelled with a variety of radionuclides was shown to depend upon the fate of the various isotopes after incorporation and more importantly after subsequent dissociation from the cells.

Contrary to one's expectations metabolically incorporated labels were least suitable for marking cells; the binding was temporary and release was faster compared with the non-specific chemically bound metallic ions. Tritium was found to be particularly unsuitable for measuring cell content in non-lymphoid tissues by assaying total radioactivity because of large scale contamination by cell-free label.

^{51}Cr was found to elute from cells at a slower rate than Uridine, Leucine or Methionine markers and had the major advantage of being efficiently excreted in urine after release thus minimizing contamination of tissues. It was therefore considered the isotope of choice to approximate the actual cell content of tissues.

^{111}In-oxine was found to be an efficient and potentially very useful label for lymphocyte migration studies both in animals and man. It was not available for the bulk of the work to be reported and has the one potential disadvantage of not being excreted on elution. However the close comparison between the results obtained with ^{111}In and ^{51}Cr supports the thesis that both isotopes reflect physiological cell behaviour. Cell-free forms of each label follow different pathways.

^{125}Udr has several disadvantages because of toxicity and elution but was found to be the most useful available label for the assay of lymphoblast content in tissues by measuring total tissue activities.

CHAPTER FOUR

The Kinetics of Tissue Localization of Radioactivity after Labelled Cell Injection - an approach to the measurement of thoracic duct cell migration in various tissues.

Introduction

The behaviour of constituent members of a population that is mobile and distributed through a multicompartmental system can be examined by tracer techniques, provided: (1) the introduction of "labelled" members does not of itself disturb the system; (2) mixing within the whole population occurs after introduction of the tracer; and (3) several compartments can be examined simultaneously after various times.

Data derived from several experimental approaches have suggested that the model of the recirculating lymphocyte pool is such a multicompartmental system, comprising mainly small long-lived lymphocytes that are in dynamic equilibrium between the blood, the secondary lymphoid organs, and the central lymph.

The model further envisages migration pathways that involve differing proportions of the population and transit times through different compartments of varying duration.

The model was built up from the following observations:

i the output of lymphocytes from the major lymphatic ducts into the blood was sufficient to replace the total blood content several times a day (Mann & Higgins 1950).

ii removal of this central lymph output via a thoracic duct fistula resulted in a profound fall

in the cell output in lymph and also depleted the cell content of the blood, the white pulp of the spleen, and the paracortical areas of lymph nodes (Mann & Higgins 1950, Gowans 1959).

iii only a small proportion of such cells in lymph had labelled DNA after continuous administration of ^{3}H Thymidine for five days and therefore the majority were relatively long-lived (Everett et al 1964).

iv reinfusion of the draining cells into the venous circulation corrected the tissue deficits and the thoracic duct output as long as the injected cells were viable (Gowans 1959).

v if these reinfused cells were "labelled" the same marked cells were found in the thoracic duct lymph after a period of hours (Gowans 1959, Shorter & Bollman 1960, Gowans & Knight 1964).

Identification of the two major traffic areas in lymph nodes and splenic white pulp by depletion studies and then by positive identification of large numbers of radio-labelled cells in autoradiographic preparations of histological sections (Gowans & Knight 1964) raised the questions of the precise anatomical pathways and time courses involved.

Information relating to lymph nodes came from two experimental approaches. Firstly, examination of histological autoradiographs a few minutes after iv injection of radio-labelled cells revealed a close association between these cells and the post-capillary venules in the paracortex - vessels which normally have a prominent lymphocytic content in their walls (Gowans & Knight 1964, Goldscheider & McGregor 1968a). Secondly, measurement of the cell content of afferent lymph and efferent lymph draining into and from a single lymph node in the sheep revealed a much higher lymphocyte concentration in the latter, together with a shift in differential cell counts such that afferent lymph contained higher proportions of large lymphocytes

and macrophages, while efferent lymph was enriched in small lymphocytes and contained very few macrophages (Hall & Morris 1965, Smith, McIntosh & Morris 1970a). Thus a net addition of small lymphocytes to the lymph in the node was indicated which was too great to be accounted for by new cell production within the node as determined by incorporation of tritiated thymidine (Hall & Morris 1965). A direct blood-lymph node-lymph pathway was thus established and the time taken to make this journey was measured by sequential collection of labelled cells in efferent lymph after their introduction into the blood. The results from both sheep and rats indicated a modal transit time of the order of 18 hrs (Ford & Simmonds 1972, Hall et al 1976, Frost et al 1975).

In the spleen the entrance to the white pulp was found by autoradiography to be via the marginal zone by direct emigration from the blood through the walls of the marginal sinuses. The use of an isolated perfused spleen preparation showed that the major exit was directly into venous blood and that the modal transit time through this compartment was approximately five hours (Ford 1969a).

Study of the distribution of radio-labelled lymphocytes within the intact animal and removal of these major sites of lymphocyte migration at various times after iv injection and assaying them for labelled cell content, either directly by autoradiography or by total tissue radioassay, showed that the content of these tissues changed with a time course that is consistent with such transit times and also gave information about the relative proportions of injected population in each compartment. (Gowans & Knight 1964, Goldschneider & McGregor 1968 a & b, Heslop & Hardy 1971, Shorter & Bollman 1960, Freitas & de Sousa 1975, Ellis & de Sousa 1975).

It is therefore reasonable to expect that examination of other compartments within the system in such kinetic distribution experiments would provide information about

the quantity and time course of lymphocyte traffic through other tissues. Several factors must be taken into account before this direct interpretation can be made without the ultimate check of examination of each tissue in isolation.

The main conditions that should be operative to allow interpretation of results in terms of cell migration through tissue are : (a) the cells labelled must be part of the total population under study - ie the recirculating lymphocyte pool - and should mix freely within that pool; (b) the "label" should be uniformly distributed among the labelled cells which should behave as a homogeneous mixture; (c) the label should be as near 'ideal' as possible in terms of elution and lack of toxicity (as discussed in the previous chapter); (d) the radioactivity assayed should be associated with viable cells which should be extravascular if true migration is to be assumed; (e) mixture within the initial compartment entered should be thorough and rapid.

The choice of thoracic duct lymphocytes for distribution studies of this type has two inherent advantages. Firstly, the population comprises virtually pure lymphocytes that are physiologically destined to enter the venous circulation, and secondly, the great majority of the cells are known to be part of the recirculating pool. However, two drawbacks are also apparent. The minority population of immunoblasts also traversing this route may not be part of the true recirculating pool and are known to have migration characteristics that differ from recirculating small lymphocytes (Gowans & Knight 1964, Hall, Parry & Smith 1972). If the whole population is labelled the contribution of this minority may influence the overall distribution of radioactivity. The contribution of this population may be to some extent evaluated by studying it alone, utilizing the specific uptake of DNA precursors by such cells. The other major complicating factor is the heterogeneity within the recirculating pool. Thus both T and B cells are known to recirculate but it is apparent

that the routes and time course through secondary lymphoid organs differ with B cells, tending to longer modal transit times (Howard 1972, Freitas & de Sousa 1976, Niewenhuis & Ford 1976). Again, non-specific labels will reflect the behaviour of a mixture of these cell types as TDL comprise very approximately 70% T cells and 30% B cells (Howard 1972, Crum & McGregor 1976). Previous studies employing uridine as a carrier for the radiolabel examined mainly T cell behaviour, as these cells take up some 10-15 times the amount of label as B cells in the rat (Howard 1972). However, the use of this label to study non-lymphoid tissue distribution is precluded by the significant contaminatory elution detailed in the previous chapter.

Because of the characteristics of the various labels available, ^{51}Cr was considered to be the most suitable even though all cell types were labelled. To gain a rough assessment of the contribution of lymphoblasts, which were probably somewhat more heavily labelled than small lymphocytes with ^{51}Cr (Eyre et al 1974), such cells were labelled in vitro with ^{125}I Udr. Furthermore, a population relatively depleted of labelled lymphoblasts was obtained by 'passaging' ^{51}Cr labelled TDL from blood to lymph in a host with an established thoracic duct fistula. Lymphoblasts recirculate into lymph poorly (Gowans & Knight 1964). It is possible that such 'passaged TDL' collected from 2-24 hrs after iv injection may also be somewhat enriched for T cells as their modal transit is somewhat shorter than that of B cells, but this possible enrichment was not quantitated.

Attempts to prepare purified populations of B or T cells were not made because of the lack of techniques available that would yield large enough numbers of cells to allow adequate labelling for distribution studies in multiple recipients. Such studies would be more feasible using ^{111}In-oxine, as much higher cell specific activities could be achieved which in turn would allow the use of many fewer cells per recipient.

Experiments were designed to measure changes in the proportion of injected radioactivity in various tissues at various times after iv injection of labelled TDL. Recipients were always subjected to whole body vascular perfusion to minimize contamination of the tissues by intravascular label. Clarification of the interpretation of the results of such experiments was provided by a series of supplementary studies. The effect of intravenous injection of cells upon that part of the recirculating population already present within the tissues of the host was studied by looking for changes in the distribution of labelled cells, that had been allowed to equilibrate with the RLP over a period of 24 hrs, upon subsequent iv injection of a large number of unlabelled cells. The use of splenectomized recipients allowed higher blood concentrations to be maintained for longer, as such an operation removes a tissue which normally accepts half of the injected cells from the circulation very quickly. It was hoped that such a manoeuvre would enable more cells to emigrate at other sites and therefore accentuate any fluctuations seen in tissue levels as suggested by the early increased lymph node localization noted in such recipients (Bradford & Born 1973).

Intra-arterial injection of labelled cells was used in an attempt to provide a known concentration of cells to peripheral tissues avoiding the systemic factors that are all working to reduce the concentration of injected labelled cells in the blood compartment so rapidly.

Autoradiographs of histological sections of all tissues studied were examined to provide qualitative confirmation of the extravasation of labelled cells at various times after iv injection. For these experiments ^{14}C or ^{3}H Udr or ^{3}H Tdr were employed as they provided much better preparations, and elution of some label does not matter in these circumstances. Very large doses of cells were sometimes used to counteract the great dilution effect within non-lymphoid tissues and increase the chances of detecting labelled

cells in the very small samples that could be examined.

METHODS

Particular techniques not included in the general methods chapter.

1 'Passaged ^{51}Cr TDL'

This population of labelled cells was included among thoracic duct cells collected during a period from 2-24 hrs after iv injection of ^{51}Cr labelled TDL into a syngeneic cannulated intermediate host. Approximately 10% of the initial dose of label was collected and the cells were washed once in PBS before resuspension in medium RPMI 1640 + 10% FCS at a concentration suitable for iv injection.

2 Splenectomy

Recipients, under ether anaesthesia, underwent splenectomy or a sham laparotomy via a left subcostal incision. The vascular pedicle of the spleen was isolated by blunt dissection and the spleen removed after a single ligature had been tied around the pedicle. The wound was closed with continuous ligatures in two layers. The operation was performed either immediately or twelve days prior to labelled cell transfer.

3 Intra-arterial Infusions

Labelled cells suspended in medium RPMI 1640 + 10% FCS or modified rat blood comprising 20% by volume syngeneic erythrocytes in plasma were infused by syringe or roller pump via a polythene cannula (PP10 Portex Ltd) with or without a 27g needle into an exposed femoral artery at the mid-femoral level in a peripheral direction. The cannulation of an artery was performed under ether anaesthesia and the vessel was ligated at the end of the infusion to prevent the otherwise inevitable haemorrhage as a relatively large incision through the arterial wall was necessary to allow introduction of

the cannula. The pressure of the infused fluid was monitored with a mercury filled U-tube manometer connected via a saline filled tube and a T connector to the infusion cannula. For experiments of short duration the anaesthesia was maintained, while for experiments with periods of 1 hr or more elapsing between the end of the infusion and tissue examination the wound was closed and the animal allowed to recover from the anaesthetic.

RESULTS

Table 4.1 summarizes the results of many experiments in which the distribution of radioactivity in most tissues of the body was examined half an hour, two hours or twenty-four hours after injection of labelled cells belonging to one of three populations:

(i) ^{51}Cr labelled whole TDL.

(ii) Passaged ^{51}Cr labelled TDL.

(iii) ^{125}I Udr labelled lymphoblasts.

The results are expressed as the percentage of the injected dose per whole organ determined as described in the previous chapter by radioassay of weighed samples obtained by dissection after whole body perfusion and calculation with reference to standard organ weights. The variation between results in triplicate recipients was much smaller within an experiment than between experiments. In the former case a standard deviation of ten percent of the arithmetic mean was usually found, but when experiments were pooled this standard deviation was nearer 50% for most tissues.

Examination of the data for "^{51}Cr whole TDL" reveals a distribution pattern in the secondary lymphoid organs similar to those reported by others (Gowans & Knight 1964, Shorter & Bollman 1960, Heslop & Hardy 1971, etc).

TABLE 4.1a THE KINETICS OF TISSUE LOCALIZATION OF RADIOACTIVITY FOLLOWING INTRAVENOUS INJECTION OF ^{51}Cr OR ^{125}I LABELLED TDLs INTO SYNGENEIC RECIPIENTS

LYMPHOID TISSUES + BODY FLUIDS % INJ DOSE/ORGAN (PERFUSED) ± S.D.

		NORMAL TDL ^{51}Cr			PASSAGED TDL ^{51}Cr			NORMAL TDL $^{125}Iudr$		
TISSUE	TIME HRS	.5	2	24	.5	2	24	.5	2	24
	wt. factor / No/group	9	10	50	4	4	5	5	10	10
SPLEEN	.4g	34.1 ± 8.8	42.1 ± 7.3	18.0 ± 4.2	46.8 ± 4.7	56.8 ± 2.2	17.1 ± 4.8	11.0 ± 1.0	9.9 ± 2.9	2.7 ± .6
SUP. L.N.'s	.5g	4.1 ± 3.1	4.7 ± 2.9	30.7 ± 13	6.5 ± 5.8	7.7 ± 1.5	25.9 ± 8.1	.88 ± .14	.75 ± .38	1.93 ± .35
MES. L.N.'s	.15g	.89 ± .44	1.52 ± .58	6.55 ± 1.8	1.35 ± 1.1	2.04 ± 1.1	6.35 ± 2.1	.39 ± .07	.76 ± .24	.66 ± .10
SMALL INTESTINE[1]	5.0g	1.77 ± .7	2.4 ± 1.2	2.42 ± 1.1	.93 ± .4	.56 ± .2	1.54 ± 1.2	13.7 ± 3.7	18.9 ± 7.5	24.9 ± 7.0
THYMUS	.3g	.013 ± .005	.014 ± .003	.073 ± .05	.006 ± .002	.008 ± .002	.026 ± .015	.067 ± .012	.063 ± .04	.082 ± .08
BONE(marrow)	10.0g	24.9 ± 7.1	25.7 ± 5.7	5.2 ± 2.5	7.9 ± 5.4	16.9 ± 7.6	2.4 ± 1.4	18.8 ± 2.9	16.4 ± 5.9	4.3 ± 2.9

[1] Excluding Peyer's Patches.

TABLE 4.1b

LYMPHOID TISSUES + BODY FLUIDS % INJ DOSE/ORGAN (PERFUSED) ± S.D.

		NORMAL TDL ^{51}Cr			PASSAGED TDL ^{51}Cr			NORMAL TDL $^{125}Iudr$		
TISSUE	TIME HRS	.5	2	24	.5	2	24	.5	2	24
	wt. factor / No/group	9	10	50	4	4	5	5	10	10
BLOOD LEUCOCYTES	15.0mls	3.26 ± 1.8	.70 ± .3	1.88 ± 1.0	15.1 ± 10.2	3.1 ± 2.1	1.7 ± .6	2.1 ± .9	.48 ± .3	.28 ± .06
WHOLE BLOOD	15.0mls	4.75 ± 1.9	1.53 ± .7	3.62 ± 2.1	13.9 ± 8.4	2.9 ± .75	2.7 ± 1.2	6.57 ± 1.9	2.19 ± 1.2	1.76 ± .4
PLASMA	9.0mls	.73 ± .3	.50 ± .20	.33 ± .09	2.54 ± .29	.16 ± .08	.12 ± .09	2.33 ± .9	1.32 ± .7	1.09 ± .38
PERFUSATE (Supernatant)	250mls	.75 ± .3	.40 ± .15	.35 ± .9	1.02 ± .6	.10 ± .05	.12 ± .3	6.88 ± 3.0	4.65 ± 1.5	2.67 ± .9
PERFUSATE (cells)	250mls	4.22 ± 5.2	.50 ± .15	1.28 ± 1.6	5.63 ± 2.5	1.48 ± .25	.73 ± .25	2.15 ± .75	.80 ± .25	.25 ± .02

TABLE 4.1c

NON-LYMPHOID TISSUES

		NORMAL TDL ^{51}Cr			PASSAGED TDL ^{51}Cr			NORMAL TDL $^{125}Iudr$		
TISSUE	TIME HRS	.5	2	24	.5	2	24	.5	2	24
	wt. factor / No/group	9	10	50	4	4	8	8	10	10
LUNG[1]	1.2g	17.3 ± 8.1	3.6 ± 1.3	2.8 ± .9	24.2 ± 12.1	3.1 ± .76	2.2 ± 1.0	34.9 ± 16.7	6.9 ± 2.9	1.3 ± .6
LIVER	10.0g	12.3 ± 3.5	11.4 ± 3.3	11.4 ± 3.8	15.8 ± 8.4	8.9 ± 2.8	9.3 ± 1.5	28.4 ± 13.0	15.9 ± 7.6	3.4 ± 1.7
KIDNEY	1.5g	.49 ± .1	.45 ± .15	.58 ± .2	.68 ± .25	.34 ± .10	.52 ± .26	.64 ± .19	.22 ± .09	.18 ± .10
SKIN	30.0g	.99 ± 3.6	.54 ± .3	.39 ± .12	1.11 ± .27	.33 ± .12	.18 ± .15	5.49 ± .9	2.49 ± .9	2.31 ± .9
MUSCLE	100.0g	1.78 ± .5	1.13 ± .8	.85 ± .5	3.29 ± 1.6	1.11 ± .5	.38 ± .2	7.21 ± 2.0	3.22 ± 1.0	2.16 ± 1.0

[1] Peripheral lung.

TABLE 4.1d

NON-LYMPHOID TISSUES

		NORMAL TDL ^{51}Cr			PASSAGED TDL ^{51}Cr			NORMAL TDL $^{125}Iudr$		
TISSUE	TIME HRS	.5	2	24	.5	2	24	.5	2	24
	wt. factor / No/group	9	10	50	4	4	8	8	10	10
OVARY	.1g	.023	± .011 ± .002	± .009 ± .004	± .020 ± .006	± .003 ± .002	± .003 ± .001	± .052 ± .007	± .021 ± .012	± .018 ± .007
UTERUS	.4g	.004	± .009 ± .008	± .008 ± .004	± .007 ± .003	± .002 ± .001	± .004 ± .001	± .179 ± .008	± .075 ± .008	± .064 ± .008
TESTIS	2.5g	± .013 ± .005	± .015 ± .005	± .025 ± .007	.049	n.d.	n.d.	± .133 ± .005	± .111 ± .04	± .092 ± .06
EPIDIDYMIS	3.5g	± .049 ± .10	± .040 ± .02	± .051 ± .003	.179	n.d.	n.d.	± .330 ± .28	± .105 ± .03	± .200 ± .14
SALIVARY GLAND	.3g	± .004 ± .003	± .004 ± .005	± .012 ± .01	± .030 ± .05	± .003 ± .001	± .017 ± .01	± .036 ± .007	± .015 ± .01	± .039 ± .07
BRAIN	1.5g	± .009 ± .006	± .006 ± .003	± .004 ± .004	± .022 ± .009	± .009 ± .001	± .003 ± .002	± .025 ± .009	± .010 ± .006	± .018 ± .03

Initially the major localization was in the spleen, which often accounted for half of the injected activity at 2 hrs. The concentration of label then fell from this peak value by a factor of two to three by 24 hrs. There was also an initial rapid localization in lymph nodes amounting to approximately 8% per hour over the first half hour in superficial nodes. This was followed by a short period of relatively slow accumulation between $\frac{1}{2}$ and 2 hrs and then by a steady and prolonged increase in activity reaching peak values around 18-24 hrs. Most lymph nodes showed very similar concentrations per unit weight with perhaps a slight tendency to higher concentrations in superficial as opposed to mesentric nodes. A notable exception was the hepatic node which almost invariably showed higher concentrations particularly at 2 hrs and 24 hrs. The extent of this increase is shown in Table 4.2.

Table 4.2

% Inj.dose/gm + SD of Lymph Node following iv Injection of ^{51}Cr whole TDL (perfused). (n) No. of recipients/group.

	$\frac{1}{2}$ hr	2 hr	24 hr
Superficial Cervical Node	8.1 ± 6.3 (4)	9.5 ± 5.8 (7)	61.4 ± 26 (24)
Mesenteric Node	5.9 ± 2.9 (9)	10.2 ± 3.9 (10)	43.7 ± 12 (24)
Hepatic Node	9.5 ± 1.4 (4)	28.4 ± 13.1 (6)	117.3 ± 69 (10)

Autoradiographs of lymph nodes removed one hour after iv injection of ^{3}H-Udr labelled TDLs confirmed this increased concentration in the hepatic lymph node and showed that many of the labelled cells were in the subcapsular sinus of this node. Cells in this compartment of lymph nodes draining other anatomical regions were very much fewer with a slightly greater frequency noted in the mesenteric node compared to superficial cervical, axillary or pre-femoral nodes (see Figs 4.1, 4.2, 4.3).

FIG 4.1

Autoradiograph of a section of mesenteric lymph node of a rat injected iv 2 hrs previously with 500 x 10^6 syngeneic TDL labelled with ^{3}H-Udr.

Many labelled cells are present in the deep cortex (lower centre field) where some are associated with specialized post-capillary venules (dilated by vascular perfusion at the time of sampling). However labelled lymphocytes are also present in the sub-capsular sinuses and in the superficial cortex between the lymphoid follicles. Labelled cells were absent from germinal centres.

Mag x 280. Methyl Green + Pyronin; green filter; 4 weeks exposure.

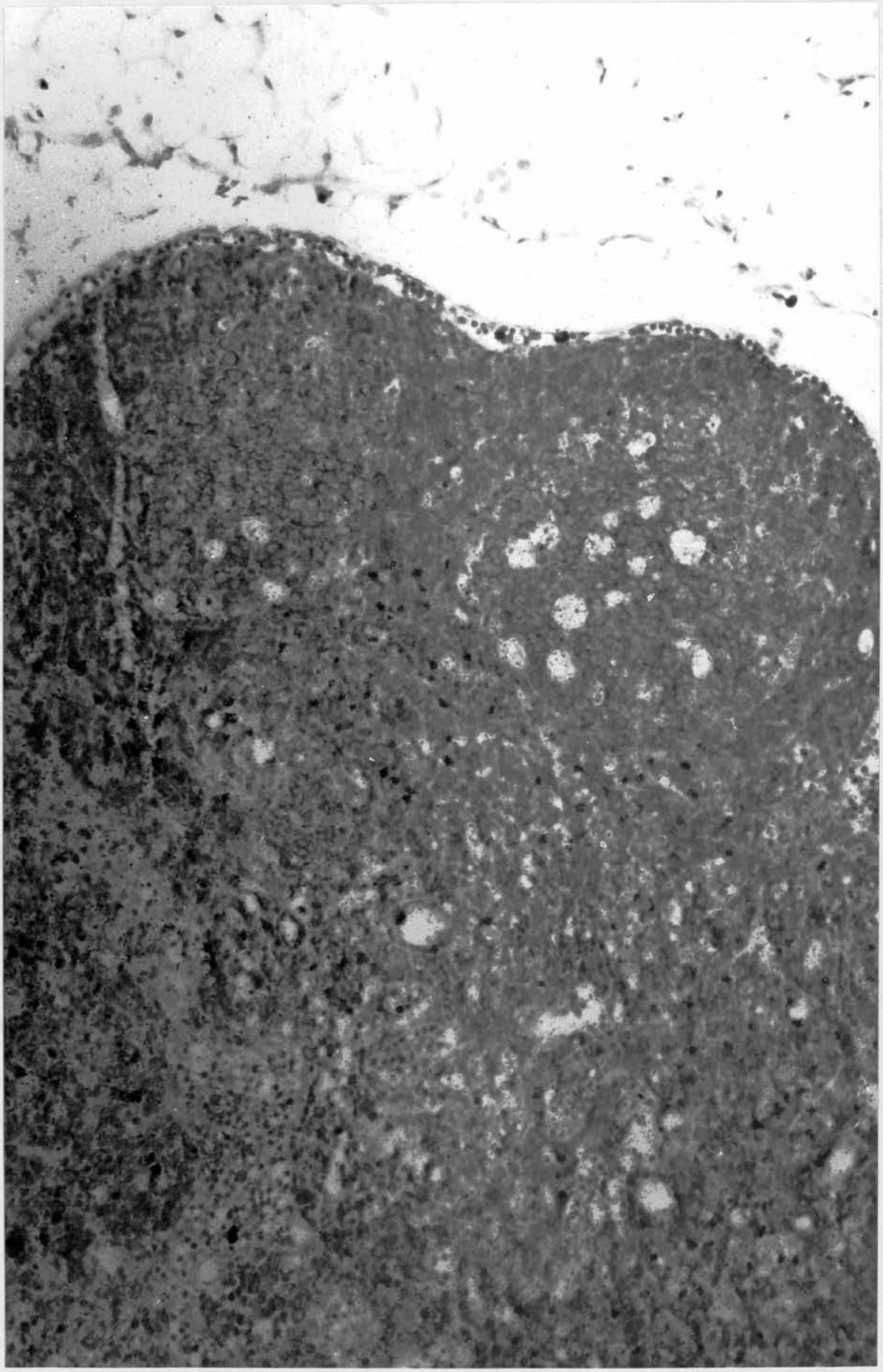


FIG 4.2

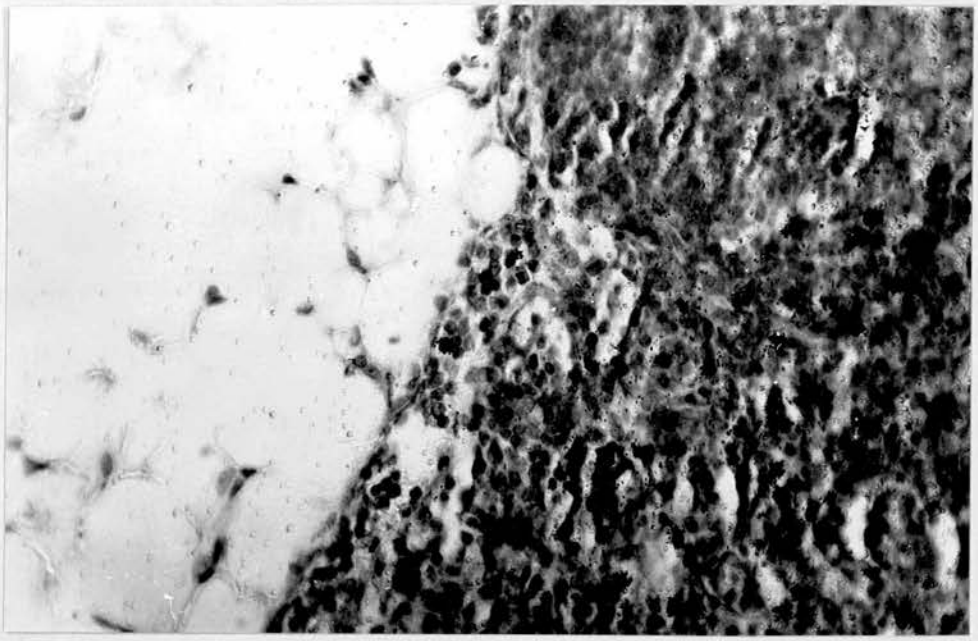
Autoradiograph of a section of Hepatic Lymph Node of a rat injected iv 2 hrs previously with 500 x 10^6 syngeneic TDL labelled with ^{3}H-Udr. Labelled lymphocytes are present in the subcapsular sinus, superficial cortex and particularly the deep- or paracortex.

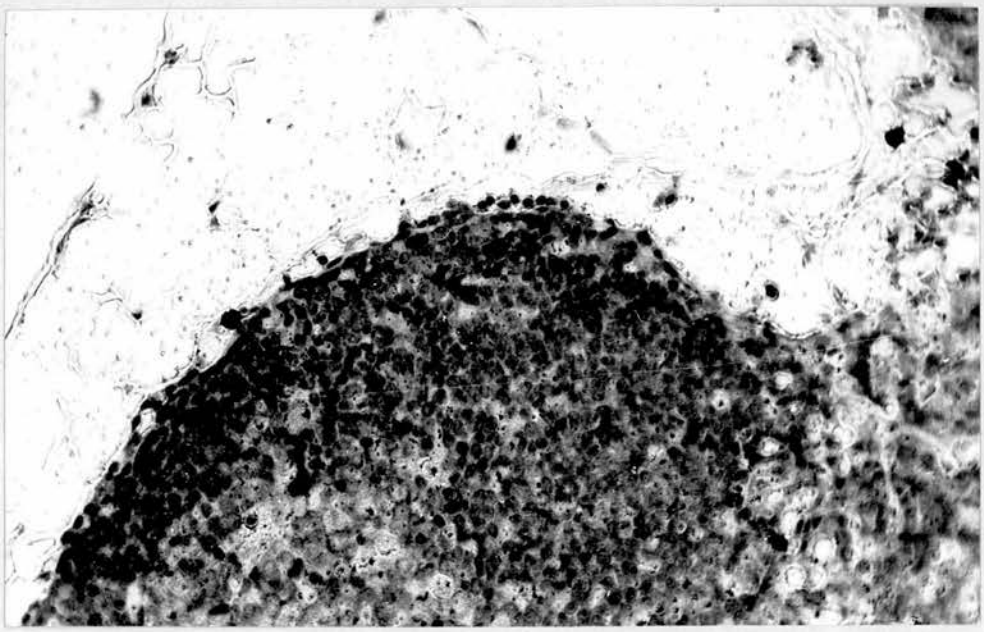
Mag x 350. Methyl Green + Pyronin; green filter; 4 weeks exposure.

FIG 4.3

Autoradiograph of a section of Hepatic Lymph Node of a rat injected iv 2 hrs previously with 500 x 10^6 syngeneic TDL labelled with ^{3}H-Tdr (in vitro).Labelled lymphoblasts are present in the subcapsular sinus and were noted sparsely distributed in the superficial and deep cortex.

Mag x 350. Methyl Green + Pyronin; green filter; 6 weeks exposure.





These results in lymph nodes and spleen act as a positive control for the techniques used and suggest the 'normal' behaviour of the injected cells. They also confirm that the choice of times at which tissues were sampled was appropriate and likely to show tissue concentrations under conditions when levels in the spleen, lymph nodes and blood were substantially different. Thus levels at half an hour will reflect the initial rate of extravasation from the blood, those at two hours will be associated with minimal blood levels and maximum spleen concentrations, while the results at 24 hrs will reflect the distribution when equilibration of the injected cells within the re-circulating lymphocyte pool is almost complete. Confirmation of this latter assumption was obtained by examining tissues in triplicate recipients perfused 24, 48, or 72 hrs after injection of aliguots of the same suspension of ^{51}Cr labelled TDL. Table 4.3 summarizes the results which show very little fluctuation between tissue concentrations at these times, allowing that some of the late decreases may be due to non-specific elution of label.

The results in other tissues showed various patterns of distribution. The small intestine excluding the organized lymphoid tissue of Peyers Patches showed significant early localization of radioactivity with a small degree of further accummulation between 2 and 24 hrs.

The thymus showed a pattern similar in shape to lymph nodes but 3 orders of magnitude less in quantity. Thus there was significant early localization by $\frac{1}{2}$ and 2 hrs (Fig 4.4) but a five fold further increase by 24 hrs, resulting in concentrations that were consistently higher than in most non-lymphoid tissues. Autoradiography of sections of thymus prepared 36 hrs after intravenous injection of ^{3}H or ^{14}C-Udr labelled TDLs showed such labelled cells located in the medullary parenchyma (see Fig 4.5, 4.6). These cells were most readily visualized after injection of large numbers (up to 3×10^{9}) of labelled cells but the proportional distribution of label

TABLE 4.3

Late Time Course of Tissue Radioactivity following iv Injection of Normal ^{51}Cr TDL into Syngeneic Recipients (Perfused).

3 recipients of the same cell suspension at each time point.

Tissue	% Inj Dose Per Gram of Tissue		
	24 hrs	48 hrs	72 hrs
Spleen	42.5	26.5	28.9
Superficial lymph nodes	65.5	53.9	49.6
Mesenteric lymph node	52.9	55.3	41.9
Small intestine	.65	.70	.51
Thymus	.37	.26	.30
Bone (marrow)	.78	.53	.49
Liver	1.4	1.2	1.4
Kidney	.33	.33	.38
Muscle	.044	.006	.007
Brain	.003	.0008	.0004
White blood cells	.153	.070	.086
Skin	.011	.010	.010

FIG 4.4

Autoradiograph of a section of thymus medulla of a rat injected iv 2 hrs previously with 500 x 10^6 syngeneic TDL labelled with ^{3}H-Udr. Labelled lymphocytes are present attached to the wall of a venule, dilated by vascular perfusion, and also in the interstitial parenchyma.

Mag x 350. Methyl Green + Pyronin; green filter; 4 weeks exposure.

FIG 4.5

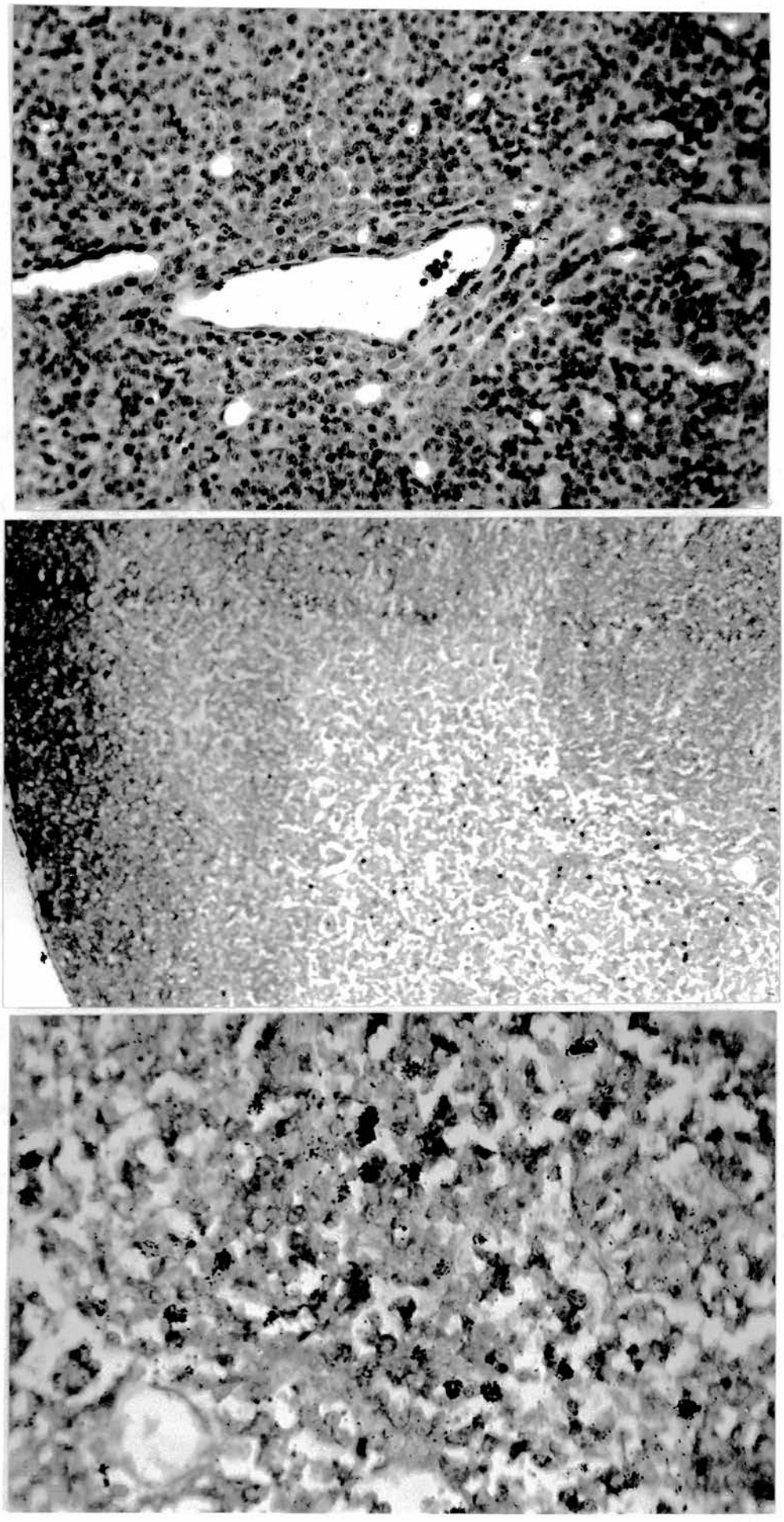
Autoradiograph of a section of thymus of a rat injected iv 36 hrs previously with 3000 x 10^6 syngeneic TDL labelled with ^{3}H-Udr. Numerous labelled lymphocytes are present in the medulla but not in the cortex.

Mag x 50. Methyl Green + Pyronin; green filter; 6 weeks exposure.

FIG 4.6

Same section as Fig 4.5. High power view of labelled lymphocytes in thymic medulla.

Mag x 500. Methyl Green + Pyronin; green filter; 6 weeks exposure.



per whole thymus did not vary with the dose of cells injected.

The bone marrow accumulated substantial proportions of radioactivity with peak values seen at 2 hrs followed by a decrease in concentration between 2 and 24 hrs of some five fold. Autoradiography of decalcified histological sections showed frequent ^{3}H-Udr labelled cells in all areas of the parenchyma with perhaps a slight tendency to an early accumulation in areas near the bone surfaces. Such labelled cells were extravascular and were very much less frequent by 24 hrs after iv injection (see Fig 4.7, 4.8). This pattern of distribution has many features in common with that seen in the spleen.

In blood a very rapid fall in concentration was characteristic. This observation and that of initial levels that are never as high as might be expected confirm those of many other workers (eg Bunting et al 1963). Bunting et al also described minimum concentrations found at around 2-4 hrs after iv injection with some small increase by 24 hrs and this observation was confirmed. The early fall in blood levels was associated with high concentrations in the lung which show rapid falls between $\frac{1}{2}$ and 2 hrs as previously reported (eg Weisburger et al 1951, Gowans & Knight 1964, Scollay & Smith 1976).

The liver accounted for about ten per cent of the injected activity at all time intervals and this lack of fluctuation was also apparent in the kidney.

The other non-lymphoid tissues studied consistently showed low total amounts of radioactivity but the concentrations were significantly above background and in general showed highest values at $\frac{1}{2}$ hr with a fall by 2 hrs and little change thereafter. However in individual experiments in which all time periods were studied with the same cell suspension a slight rise in concentrations - eg in skin - was often seen between 2 and 24 hrs. Such slight changes

FIG 4.7

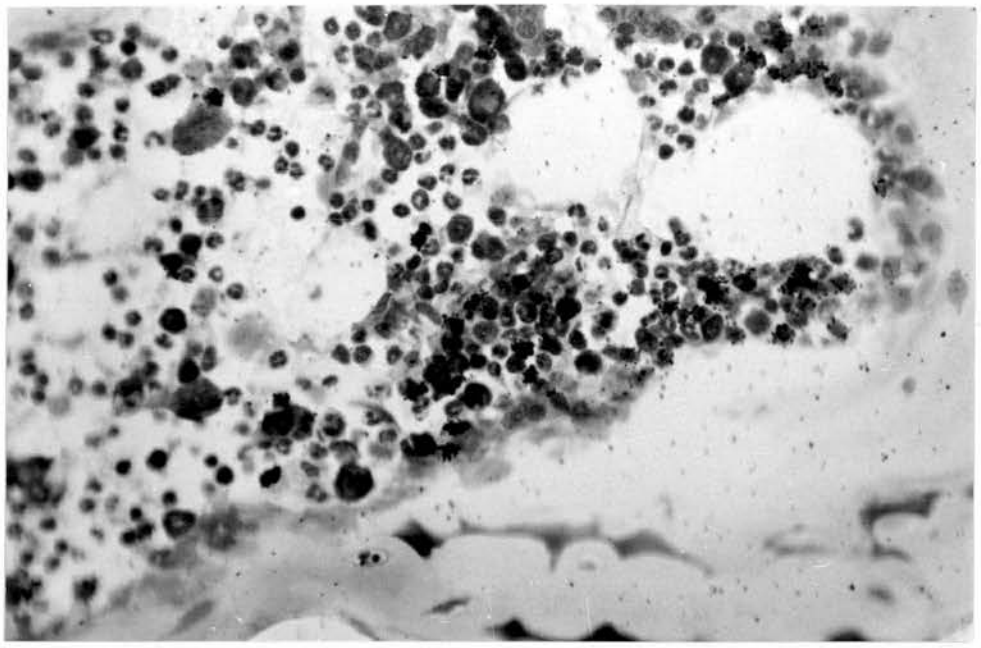
Autoradiograph of a section of decalcified bone and marrow from a rat injected 2 hrs previously with 500 x 10^6 syngeneic TDL labelled with ^{3}H-Udr. Many labelled lymphocytes are present in the marrow parenchyma particularly in areas close to bone surfaces. The sinuses are widely dilated by vascular perfusion at the time of sampling.

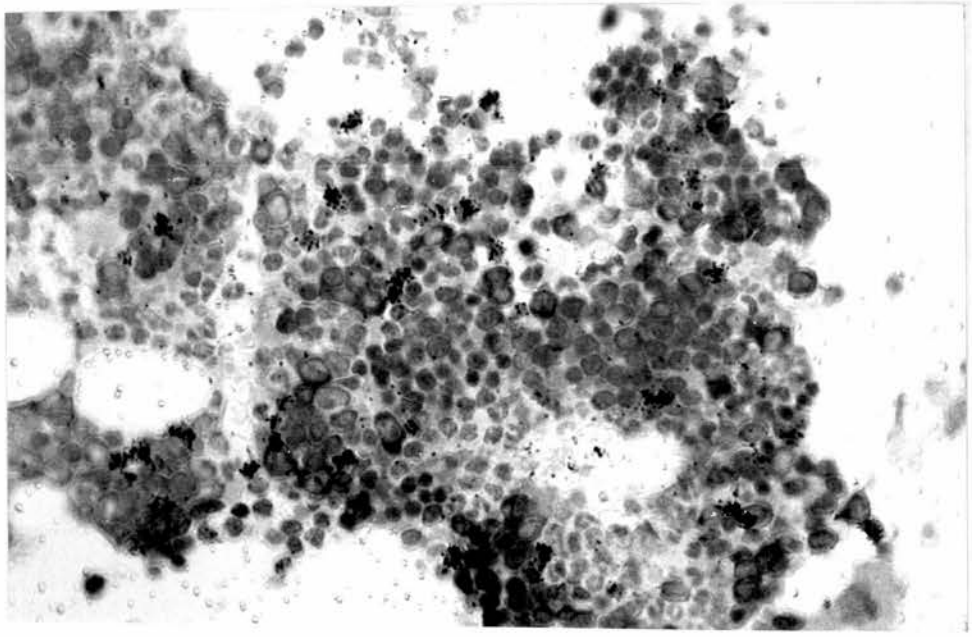
Mag x 200. Methyl Green + Pyronin; green filter; 4 weeks exposure.

FIG 4.8

Autoradiograph of a section of a 'plug' of bone marrow expelled from the femur of the same animal as Fig 4.7. Numerous labelled lymphocytes are intimately mixed with unlabelled marrow cells.

Mag x 240. Methyl Green + Pyronin; green filter; 4 weeks exposure.





cannot be seen in the pooled data from multiple experiments, because of the wider interexperiment variation. At equilibrium non-lymphoid tissues had concentrations approximately $^{1}/_{10,000}$ of those in lymph nodes. Extensive searches of histological autoradiographs confirmed the presence of labelled cells in all tissues; Figs 4.9, 4.10, 4.11, show examples of interstitial labelled lymphocytes in brain and testis.

'Passaged ^{51}Cr TDL' produced a distribution pattern that was usually very similar to that of the whole population labelled with ^{51}Cr. It was noteworthy that there was significant localization of radioactivity in the small intestine and thymus which both also showed some late accumulation.

The bone marrow again showed substantial but temporary early localization, as did the spleen. Non-lymphoid tissues showed if anything a more pronounced 'peak' at $\frac{1}{2}$ hr, partly as a result of more substantial falls by 2 and 24 hrs. The minimum blood leucocyte concentration at two hrs was consistently absent with this population.

^{125}I Udr-labelled lymphoblasts

The tissue distribution pattern following the iv injection of this population differed markedly from that of the other two populations, as expected. The proportionately smaller localization in the spleen and lymph nodes confirmed the observations of others (Gowans & Knight 1964; Hall, Parry & Smith 1972), as did the substantial localization in the small intestine at both early and late intervals. An increased concentration in the mesentric lymph node when compared to superficial nodes (eg cervical) was noted at 2 hrs but not at 24 hrs. As with the other populations, significant activity was found in the thymus. The bone marrow accounted for a large proportion of the injected activity with a similar time course as seen with ^{51}Cr.

Concentrations in lung and blood both fell rapidly from their initial levels and it was noted that the majority of

FIG 4.9

Autoradiograph of a section of brain of a rat injected iv 24 hrs previously with 1080 x 10^6 syngeneic TDL labelled with ^{14}C -Udr. Two labelled lymphocytes are present in the interstitium and the dilated capillary shows the effect of vascular perfusion at the time of sampling.

Mag x 450. Methyl Green + Pyronin; green filter; 6 weeks exposure.

FIG 4.10

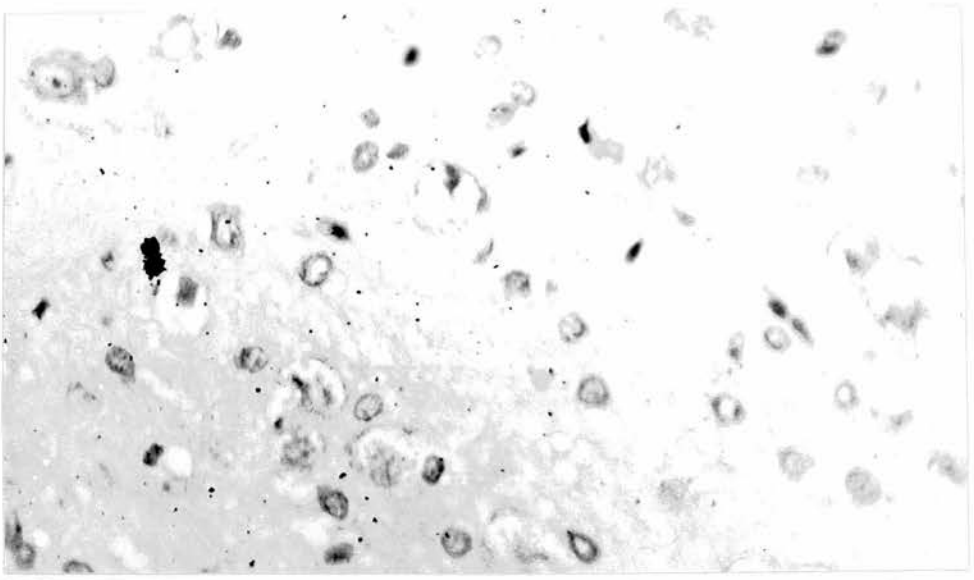
Same animal as Fig 4.9. A labelled lymphocyte in the interstitium of the cerebellum.

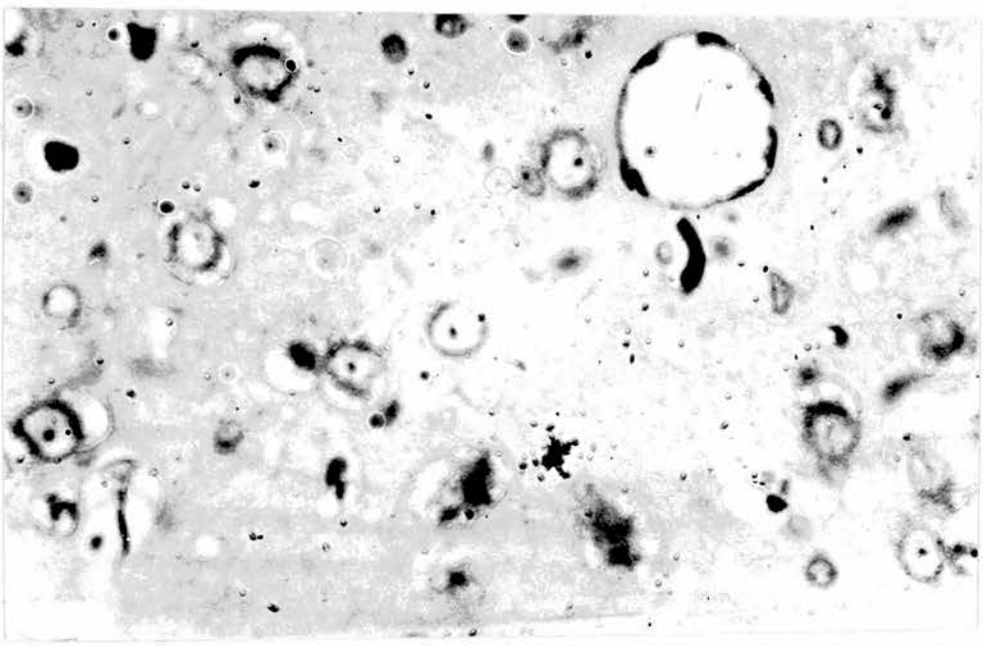
Mag x 550. Methyl Green + Pyronin; green filter; 6 weeks exposure.

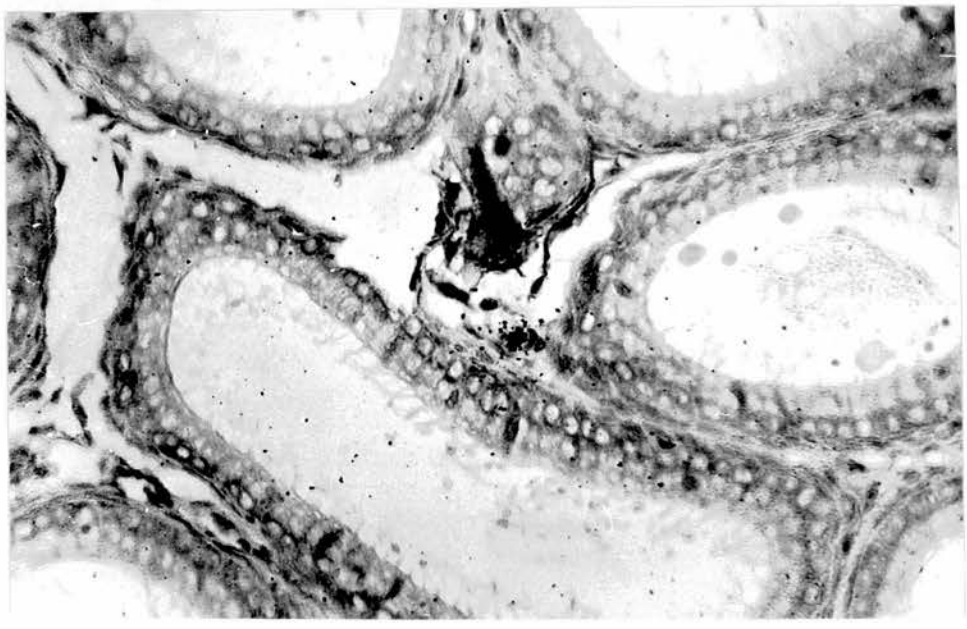
FIG 4.11

Autoradiograph of a section of testis from a rat injected iv 24 hrs previously with 215 x 10^6 syngeneic TDL labelled with ^{14}C-Udr. The recipient was perfused at the time of sampling. A labelled lymphocyte is seen in the interstitial connective tissue.

Mag x 150. Methyl Green + Pyronin; green filter; 6 weeks exposure.







the activity in blood was in the cell free plasma fraction.

The liver accounted for a considerable proportion of the label initially but this level fell steadily over 24 hrs unlike the ^{51}Cr activity. However, the total accountability of ^{125}I was also much lower than for ^{51}Cr by 24hrs.

The consistent excess of ^{125}I in the supernatant perfusate over that in the total plasma volume showed that some contamination of tissues by eluted label was likely. Non-lymphoid tissues which showed maximum levels at $\frac{1}{2}$ hr with subsequent falls by 2 hrs and 24 hrs consistently showed proportional concentrations that were several times greater than those seen with either ^{51}Cr population.

In an attempt to accentuate these possible early fluctuations in non-lymphoid tissue concentrations the distribution of labelled cells in splenectomized recipients was studied at similar time intervals.

Data pooled from several experiments is shown in Table 4.4, in which the results are expressed as tissue concentrations - ie arithmetic mean percentage injected dose/gram of tissue perfused at $\frac{1}{2}$, 2 or 24 hrs after iv injection of ^{51}Cr labelled cells. The results which confirm those of Bradfield & Born (1973) showed at least double intact control activities in lymph nodes of splenectomized recipients at $\frac{1}{2}$ and 2 hrs but little difference at 24 hrs. This extent of increase was measured in most tissues, being most obvious in blood, lungs and bone marrow, and least noticeable in non-lymphoid tissue such as skin.

Table 4.5 shows the results of experiments in which the recipients had been allowed 12 days to recover from the effect of the surgical trauma of splenectomy or sham laparotomy. The distribution of label at 2 hrs and 24 hrs confirms the results shown in Table 4.4, showing that the differences are due to the lack of the spleen which acts to remove large numbers of lymphocytes from the circulation soon after injection, rather than a non-specific effect

TABLE 4.4 THE KINETICS OF TISSUE LOCALIZATION OF ^{51}Cr AFTER I.V. INJECTION OF LABELLED TDLs INTO SYNGENEIC INTACT OR SPLENECTOMIZED RECIPIENTS

% INJ DOSE/GM OF TISSUE (PERFUSED) ± S.D.

	½HR		2HRS		24HRS	
TISSUES	INTACT (n = 9)	SPLX (n = 2)	INTACT (n = 10)	SPLX (n = 4)	INTACT (n = 50)	SPLX (n = 13)
SPLEEN	85.1 ± 22		105.2 ± 18		45.1 ± 10.5	
SUP. L.N.'s	8.1 ± 6.3	33.5 ± 17.5	9.5 ± 5.8	30.1 ± 15.7	61.4 ± 26.0	80.0 ± 41.8
MES. L.N.'s	5.9 ± 2.9	10.2 ± 5.2	10.2 ± 5.2	22.8 ± 9.1	43.7 ± 12.2	63.5 ± 21.1
SMALL INT.	.35 ± .14	.89 ± .28	.48 ± .25	.75 ± .12	.48 ± .22	1.26 ± .91
THYMUS	.044 ± .02	.084 ± .02	.047 ± .01	.076 ± .01	.245 ± .2	.375 ± .10
BONE (marrow)	2.5 ± .71	4.78 ± 2.9	2.6 ± .6	7.09 ± 2.7	.52 ± .25	1.47 ± .49
BLOOD LEUC. (/ml)	.22 ± .12	.86 ± .48	.05 ± .02	.15 ± .01	.13 ± .07	.12 ± .04
LUNG	14.5 ± 6.8	40.4 ± 15.2	3.0 ± 1.1	9.4 ± 4.7	2.3 ± .81	2.7 ± 1.1

TABLE 4.4 contd.

% INJ DOSE/GM OF TISSUE (PERFUSED) ± S.D.

	½HR		2HRS		24HRS	
TISSUES	INTACT (n = 9)	SPLX (n = 2)	INTACT (n = 10)	SPLX (n = 4)	INTACT (n = 50)	SPLX (n = 13)
LIVER	1.23 ± .35	2.35 ± .09	1.14 ± .33	2.0 ± .54	1.14 ± .38	1.62 ± .37
KIDNEY	.32 ± .07	1.33 ± .26	.30 ± .10	.40 ± .05	.39 ± .14	.43 ± .05
OVARY	.23	1.15	.11 ± .02	.21 ± .09	.09 ± .04	.12 ± .06
UTERUS	.012	.017	.025 ± .02	.015 ± .002	.021 ± .01	.033 ± .015
TESTIS	.005 ± .002	.012	.006 ± .002	.006	.010 ± .003	.012 ± .004
EPIDIDYMIS	.014 ± .031	.076	.012 ± .007	.030	.015 ± .001	.041 ± .018
MUSCLE	.018 ± .005	.031 ± .012	.011 ± .008	.014 ± .004	.008 ± .005	.015 ± .005
BRAIN	.006 ± .004	.018 ± .006	.004 ± .004	.015 ± .004	.003 ± .003	.004 ± .003
SKIN	.033 ± .012	.041 ± .001	.018 ± .01	.027 ± .004	.013 ± .004	.012 ± .003

TABLE 4.5 THE KINETICS OF TISSUE LOCALIZATION OF ^{51}Cr AFTER I.V. INJECTION OF ^{51}Cr LABELLED TDLs INTO SPLENECTOMIZED OR SHAM OPERATED RECIPIENTS

ARITHMETIC MEAN (of 2) % INJ DOSE/GM OF TISSUE (PERFUSED)

	2 HRS		20 HRS	
TISSUES	SHAM OP.	SPLX	SHAM OP.	SPLX
SPLEEN	121.8 ± 2.7		44.1 ± .001	
SUP. LYMPH NODES	11.7 ± 1.9	18.8 ± 4.2	55.2 ± .64	48.0 ± 4.9
MES. LYMPH NODES	8.9 ± .3	23.3 ± 3.9	44.4 ± .78	41.4 ± 12.1
HEPATIC LYMPH NODE	38.6 ± 20.5	38.5 ± 3.3	69.1 ± 1.5	54.4 ± 5.3
SMALL INTESTINE	.71 ± .05	.65 ± .003	.73 ± .35	.59 ± .02
THYMUS	.06 ± .006	.08 ± .006	.199 ± .03	.31 ± .01
BONE (marrow)	2.84 ± .21	5.11 ± 1.2	.69 ± .11	.83 ± .21
BLOOD LEUCOCYTES	.041 ± .01	.141 ± .02	.121 ± .02	.119
LUNG	2.31 ± .1	5.4 ± .64	2.2 ± .51	1.3 ± .27
LIVER	1.46 ± .1	2.39 ± .51	1.09 ± .04	1.83 ± .007
KIDNEY	.37 ± .1	.44 ± .03	.38 ± .02	.45 ± .06
OVARY	.122 ± .001	.158 ± .06	.039 ± .003	.088 ± .002
UTERUS	.034 ± .02	.016 ± .001	.012 ± .002	.024 ± .006
MUSCLE	.006 ± .004	.012 ± .003	.006 ± .001	.009 ± .002
BRAIN	.003 ± .001	.012 ± .001	.001 ± .001	.004 ± .002
SKIN	.015 ± .009	.025 ± .005	.014 ± .006	.011 ± .001

following surgery. As this "sink" only removes about ten percent of ^{125}I Udr labelled lymphoblasts in normal recipients, the effect of such an operation on this population is likely to be less pronounced, there being fewer cells made available for redistribution. This prediction is confirmed by the results shown in Table 4.6, which shows the distribution of a mixture of passaged ^{51}Cr-labelled TDL and ^{125}I Udr-labelled lymphoblasts in recipients one hour after iv injection. Not only are there smaller differences between tissue concentrations of ^{125}I in intact and splenectomized recipients but also the ratio of $^{125}I/^{51}Cr$ in each tissue tends to be smaller in the splenectomized animal. Similar results for ^{125}I were found at $2\frac{1}{2}$ hrs (Table 4.7). In general the gut showed the greatest increase in ^{125}I and as with ^{51}Cr differences in non-lymphoid tissue were least dramatic.

The most important observation from these experiments was the correlation between the increased blood leucocyte concentration at $\frac{1}{2}$ and 2 hrs in splenectomized recipients with a similar increase in lung concentrations. Previous interpretations of the initial high localization of labelled cells in the lung of normal animals had suggested a mechanism involving sequestration or embolization of cells in lung vessels as a result of possible changes to the lymphocytes due to in vitro manipulation (Gowans & Knight 1964, Hall Scollay & Smith 1976). The present results make such an explanation untenable as there is no way in which the removal of the spleen could slow the rate of decline in lung levels without the cells being mobile within the blood circulation and not sequestered. This pattern of distribution in the lung would however result if lymphocytes normally traversed the lung capillary bed relatively slowly so that the localization is apparent rather than real and follows the rapid return of labelled cells (which have made a circuit with the blood during which they failed to extravasate elsewhere) to this capillary bed.

TABLE 4.6

THE DISTRIBUTION OF RADIOACTIVITY ONE HOUR AFTER I.V. INJECTION OF PASSAGED ^{51}Cr TDLs AND ^{125}IUDR LABELLED TDLs INTO INTACT OR SPLENECTOMIZED RECIPIENTS.

% INJ DOSE/GM OF TISSUE AND RATIO OF % INJ ^{125}I/% INJ ^{51}Cr.(R)

TISSUE	INTACT			SPLENECTOMIZED		
	$p^{51}Cr$	^{125}I	R	$p^{51}Cr$	^{125}I	R
SPLEEN	131.1	24.3	.18			
SUP. LYMPH NODES	33.0	1.8	.05	55.5	2.03	.04
MES. LYMPH NODES	21.6	3.3	.15	28.0	2.9	.10
SMALL INTESTINE	.32	1.94	6.0	.67	2.7	4.0
THYMUS	.29	.22	.7	.09	.27	3.0
BONE MARROW	1.3	1.8	1.4	3.9	2.3	.6
BLOOD LEUCOCYTES	.27	.09	.33	.86	.07	.1
LUNG	8.9	32.8	3.7	32.7	41.2	1.2
LIVER	.78	2.7	3.5	1.7	3.26	1.9
KIDNEY	.26	.55	2.0	.73	.79	1.1
TESTIS	.02	.05	2.5	.01	.05	6.0
EPIDIDYMIS	.05	.16	3.0	.09	.18	2.0
BRAIN	.016	.026	1.6	.06	.05	.8
SKIN	.03	.21	6.9	.05	.20	4.3

TABLE 4.7

THE DISTRIBUTION OF ^{125}I $2\frac{3}{4}$ HOURS AFTER I.V. INJECTION OF $^{125}IUdr$ LABELLED LYMPHOBLASTS IN TDLs INTO INTACT OR SPLENECTOMIZED RECIPIENTS.

% INJ DOSE/GM OF TISSUE

TISSUE	INTACT	SPLENECTOMIZED
SPLEEN	17.6	
SUP. LYMPH NODES	.88	1.3
MES. LYMPH NODES	3.5	3.7
SMALL INTESTINE	2.9	4.2
THYMUS	.27	.26
BONE MARROW	1.1	2.1
BLOOD LEUCOCYTES	.014	.027
LUNG	5.3	6.2
LIVER	1.6	2.0
KIDNEY	.13	.15
OVARY	.05	.26
UTERUS	.03	.05
MUSCLE	.02	.04
BRAIN	.005	.006
SKIN	.06	.05

The initial precipitous fall in labelled leucocyte concentration in the blood after injection of a bolus of cells might suggest that there is a homeostatic mechanism regulating the lymphocyte content of blood. If such a mechanism existed it should act upon the lymphocytes within the host as well as any artifically added to the blood compartment. Experiments designed to detect such a mechanism were carried out by preloading recipients with ^{51}Cr labelled TDL which were allowed 24 hrs in which to equilibrate within the recirculating pool. Such recipients were then "challenged" with a large 'bolus' of unlabelled TDL, an equivalent volume of cell free fluid or left untreated as controls. Half an hour later the recipients were subjected to whole body perfusion as before and the proportional distribution of the original ^{51}Cr labelled cells was measured with particular emphasis on tissues where such homeostatic mechanisms might exist - viz lung, spleen and bone marrow.

Table 4.8 shows the results of such an experiment expressed as the arithmetic mean (of duplicate recipients) percentage injected ^{51}Cr/organ.

Allowing for the inherent variation between recipients, no differences can be detected with any of the treatments, suggesting that the injected cells behave independently and do not interact with those already present in the host. This further suggests that the rapid lowering of blood concentration of labelled cells is simply a reflection of the rapid rate of extravasation and therefore short half-time within the blood compartment. Support for this hypothesis comes from the lack of difference seen in the proportional distribution of label carried on a wide range of cell doses (10×10^6 - 3×10^9) which might otherwise be expected to have a variable effect upon any putative homeostatic control.

The distribution following intra-arterial injection of labelled cells into a peripheral artery allowed further

TABLE 4.8 DISTRIBUTION OF ^{51}Cr AFTER I.V. INJECTION OF ^{51}Cr

TDLs. LABELLED CELLS INJECTED - 24 HRS; RECIPIENTS TREATED BY INJECTION OF A) 500×10^6 UNLABELLED TDLs; EQUAL VOLUME OF PBS: OR NIL AT TIME ZERO.

TISSUES SAMPLED AT + $\frac{1}{2}$ HR.

ARITHMETIC MEAN % INJ DOSE/ORGAN (PERFUSED) S.E. approx. 10% of mean for most tissues. $n=3$

TISSUES	500×10^6 UNLABELLED TDLs	NIL	2.0 mls PBS (cell free)
SPLEEN	18.5	18.1	17.8
SUP. LYMPH NODES	8.1	8.7	7.6
MES. LYMPH NODES	5.4	5.7	6.5
SMALL INTESTINE	1.9	2.9	2.1
THYMUS	.051	.057	.071
BONE (marrow)	4.9	3.4	4.5
BLOOD LEUCOCYTES	3.1	2.1	1.8
LUNG	2.4	2.3	2.6
LIVER	4.9	5.7	7.1
KIDNEY	.46	.50	.46
MUSCLE	.39	.69	.42
SKIN	.16	.31	.08

examination of the initial localization of labelled cells in non-lymphoid tissues, to define whether the early 'peak' was real and not just an artefact resulting from incomplete wash-out of labelled cells from the intravascular compartment. Under these conditions some peripheral tissues could be supplied with a known concentration of labelled cells maintained over a period and information relating to the rate of extravasation could become available. The complete removal of interacting factors can be achieved only in isolated perfused organ models but such isolation is not feasible with diffuse tissues such as skin.

Table 4.9 summarizes the results of several experiments in which ^{51}Cr labelled cell suspensions were infused into the arterial circulation. Interpretation of the results must take into account the necessity of ligating the artery used for infusion to prevent inevitable haemorrhage upon removing the cannula. This implies a reliance upon collateral circulations both during the period of the experiment and for the saline perfusion.

Experiment 101 involved the measurement of tissue distribution after whole body perfusion at several times after a seven minute infusion of ^{51}Cr labelled TDL into one femoral artery at the mid-femoral level. The concentration, rate of administration and pressure were approximately physiological. The results in the tissues of the directly perfused leg show highest concentrations ten minutes after the end of the infusion with decreasing concentrations at 1 and 2 hrs except in the marrow, which showed a higher value at 2 hrs than at 1 hr. A similar pattern is seen in the tissues of the contra-lateral leg but with lower concentrations. Even lower concentrations were measured in similar tissues from the trunk of the animal with much less time dependent variation. It was notable that the variation between the concentration in bone marrow from various sites was not great. The lung consistently showed high concentrations soon after ia injection of the same

TABLE 4.9

THE DISTRIBUTION OF ^{51}CR AFTER LABELLED TDL INJECTION INTO THE ARTERIAL CIRCULATION OF SYNGENEIC RECIPIENTS

% INJ DOSE/GM TISSUE (PERFUSED)

		EXPERIMENT 101 INT. ART. INFUSION OF 7mins DURATION INTO FEMORAL ARTERY			EXPERIMENT 100 INT. ART. INFUSION OF 5.5mins DURATION INTO FEMORAL ARTERY		EXPERIMENT 88 INT. ART. INTO LOWER ABDOMINAL AORTA OVER 5mins	EXPERIMENT 96 INFUSION INTO FEMORAL ARTERY OVER ONE HOUR
		10min	1hr	2hr	15min	1hr	HALF HOUR	5mins
DIRECTLY PERFUSED LEG	SKIN	.28	.07	.02	1.9	2.0	.03	.87
	MUSCLE	.86	.06	.05	1.4	.11	.06	.32
	BONE (Tibia-Marrow)	1.47	.58	1.58	1.5	1.4	.21	2.3
CONTRALATERAL LEG	SKIN	.13	.10	.12	4.6	3.4		.03
	MUSCLE	.03	.01	.04	.24	.10		.02
	BONE (Tibia-Marrow)	1.31	1.68	1.07	.50	1.2		.63
TRUNK	SKIN	.08	.02	.02			.03	.07
	MUSCLE	.04	.02	.04			.05	.05
	BONE (Humerus-Marrow)	.94	1.37	.83			.23	
	LIVER	2.8	.64	.48	1.8	1.3	3.1	1.1
	SPLEEN	27.9	63.9	44.2	10.1	31.3	6.7	14.6
	LUNG	19.8	2.2	1.1	32.5	2.9	40.9	12.6
	BLOOD LEUCOCYTES	6.4	1.4	1.0				

order of magnitude as after iv injection, confirming the observations of Weisburger et al (1951). The spleen also contained substantial radioactivity soon after injection.

Experiment No.100 also showed relatively small differences between the concentrations in tissues of the directly perfused and contralateral limbs, and in Experiment No.88, in which tissues were sampled half an hour after injection into the lower abdominal aorta, there was again little difference between skin and muscle in the legs or from the flanks of the upper half of the body, which were presumed not to receive the infusion direct. The highest absolute values in peripheral tissues were found immediately after the end of an hour-long infusion of a labelled cell suspension. These results all suggested that the rate of extravasation in non-lymphoid tissues was low and that the concentrations of labelled cells supplied directly was not much, if any, less than after iv injection, supporting the idea that in the latter situation thorough mixing in the blood pool did occur quickly and all the injected cells were available to the systemic circulation.

The possible inefficiency of wash-out by whole body perfusion may still be raised as a criticism, particularly at very short intervals after injection, and this factor can never be completely removed. Evidence in support of the idea that the remaining intravascular cells are unlikely to account for all the tissue activity measured is (i) the ability to perfuse some highly vascular tissues - eg brain - to an extent that they give lower concentrations than elsewhere - eg skin - even at early intervals when the blood activity is high; (ii) by 2 hrs the concentration of ^{125}I was increased in irritated skin but that due to ^{51}Cr in the same animal decreased between $\frac{1}{2}$ and 2 hrs. In the blood the concentration of both isotopes was decreasing over this time period; (iii) most of the activity estimated for the total blood volume can be accounted for in the blood sample and total perfusate; (iv) previous reports using ^{51}Cr labelled red cells show

very low residual red cell content of tissues after simple "bleeding out" - eg Skin 2.2ul rbc/gram, and Muscle 3.6ul rbc/gram, in 200 gm rats (Meulheims et al 1959). If the total red blood cell volume was 4.4ml, then for skin .05% of the total blood activity would be present per gram of skin, which, if the ^{51}Cr leucocyte data at 24 hrs are considered, would result in a figure of approx. $\frac{.05}{100}$ x 1.88 = .0009% of the initial dose of ^{51}Cr labelled TDL. In an experiment of similar design ^{51}Cr labelled red cells were injected iv into syngeneic recipients. 24 hrs later two recipients were exsanguinated by section of the abdominal aorta while two others were subjected to whole body perfusion. The results of tissue distribution (Table 4.10) expressed as mean percent inj.dose/gm, show that the exsanguination gives results that are comparable to those reported by Meulheims and in most tissues "perfusion" lowers the residual activity by a further factor of approximately 50%. Thus if the total blood ^{51}Cr activity is taken as the equivalent of the total injected red cell dose in experiments using labelled lymphocytes, the maximum activity that can be accounted for by residual blood is likely to be, eg in skin, .001% inj.dose/gm. This is in fact less than $^{1}/10$th of the activity measured and assuming that lymphocyte behaviour within the circulation is somewhat similar to that of erythrocytes, it suggests that the activity measured reflects true extravasation. This is supported by the results of an experiment in which the "perfusate" of a normal animal was collected in sequential 20ml fractions and each fraction was analysed for total leucocyte count. The total leucocyte output fell steeply and then more gradually in successive fractions and the total found approximated the estimate made of the total blood content from counts made on a cardiac blood sample (Table 4.11).

The other possible way of measuring interstitial lymphocyte flux directly is to collect the output of afferent lymph from various tissues. This approach is precluded in

TABLE 4.10

DISTRIBUTION OF RADIOACTIVITY 24 HR AFTER ^{51}Cr LABELLED ERYTHROCYTES INJECTED I.V. INTO SYNGENEIC RECIPIENTS. COMPARISON OF EXSANGUINATION AND WHOLE BODY PERFUSION.

MEAN % INJ DOSE/GM OF TISSUE ± S.D.

TISSUES	EXSANGUINATED	PERFUSED	% of remaining activity after perfusion compared to exsanguination
SPLEEN	3.4 ± .08	3.26 ± .51	96
LYMPH NODE	.047 ± .002	.019 ± .006	41
SMALL INTESTINE	.103 ± .024	.013 ± .0002	12
THYMUS	.063 ± .001	.026 ± .004	40
BONE MARROW	.209 ± .028	.083 ± .015	40
LUNG	1.71 ± .24	1.24 ± .14	72
LIVER	.429 ± .117	.151 ± .004	35
KIDNEY	.603 ± .128	.235 ± .036	39
OVARY	.188 ± .039	.081 ± .039	43
UTERUS	.082 ± .011	.019 ± .003	24
MUSCLE	.078 ± .017	.024 ± .011	31
BRAIN	.083 ± .002	.003 ± .0002	3
SALIVARY GLAND	.089 ± .011	.011 ± .010	12
SKIN	.045 ± .012	.019 ± .006	42
BLOOD	8.9 ± .91	8.6 ± .35	
PLASMA	.016 ± .001	.032 ± .008	
TOTAL RECOVERY	⌓ 65%	⌓ 80%	

TABLE 4.11

The Effectiveness of Whole Body Perfusion in the Removal of Intravascular Nucleated Cells.

Sequential 25ml Collections of Vascular Perfusate - Total Nucleated Cell Count in Zaponim™ Treated Samples Measured in Coulter Electronic Particle Counter.

Sample No.	Cell Count x 10^6	
1	42.6	
2	6.9	
3	7.1	(71% lymphocytes)
4	7.7	
5	5.9	
6	5.3	
7	4.0	(60% lymphocytes)
8	5.4	
9	4.7	
10	6.8	
11	3.5	
12	3.6	
13	3.6	
14	3.6	
15	5.1	
16	2.6	
17	2.4	
18	1.7	
Total Recovered	122.5 x 10^6	
Total Estimated in Blood	161 x 10^6	(57% lymphocytes)
% Recovery	76%	

the rat because the ducts are too small to cannulate. A much less satisfactory approach is to examine autoradiographs of tissues and lymph nodes for labelled cell content in extravascular sites and in afferent lymphatic channels. The only place to be sure of identifying the latter structures is at their entrance to lymph nodes - ie the subcapsular sinuses.

Tissues were examined at $\frac{1}{2}$, 1, 2, or 24 hrs after iv injection of large doses of ^{3}H Udr or ^{14}C Udr labelled TDL or ^{3}H Tdr in vitro labelled lymphoblasts.

At one hour after injection of labelled small lymphocytes many labelled cells were found in the paracortical areas of all lymph nodes with many cells associated with the post-capillary venules. Labelled cells were also seen in the subcapsular sinus of some nodes. This occurrence was most frequent in the hepatic node, common in the mesenteric node, and infrequent in superficial cervical, axillary or prefemoral nodes. In addition, labelled cells were also found in interstitial lymphatic ducts in the immediate vicinity of the hepatic node at this time. Examination of similar tissues at one hour after injection of the same total number of cells containing ^{3}H Tdr-labelled lymphoblasts showed a much lower frequency of lymphoblasts in the paracortex of all nodes. These were most frequent in the mesenteric node in which many labelled blasts were also found in the subcapsular sinuses. It was impossible to decide whether the total increase in frequency could be accounted for by the entrance of cells from the afferent lymph or whether some degree of specificity of entry according to tissue of origin was also operative at the post-capillary venule. The hepatic node again showed a high overall frequency with many labelled cells in the subcapsular sinus. The peripheral nodes contained only infrequent labelled cells.

At $\frac{1}{2}$ hr, all nodes showed labelled cells only in association with post-capillary venules in the paracortex. By 24

hrs the distribution within nodes was much more diffuse and occasional cells could be found in the subcapsular sinus of all types of node.

At 24 hrs occasional cells labelled with either ^{3}H or ^{14}C Udr were identified in the interstitium of all tissues by autoradiography of tissue sections including brain after large numbers of labelled cells had been injected. As already mentioned, labelled cells were found in thymic medulla and bone marrow at frequencies that confirmed the distribution pattern seen with whole organ radioassay.

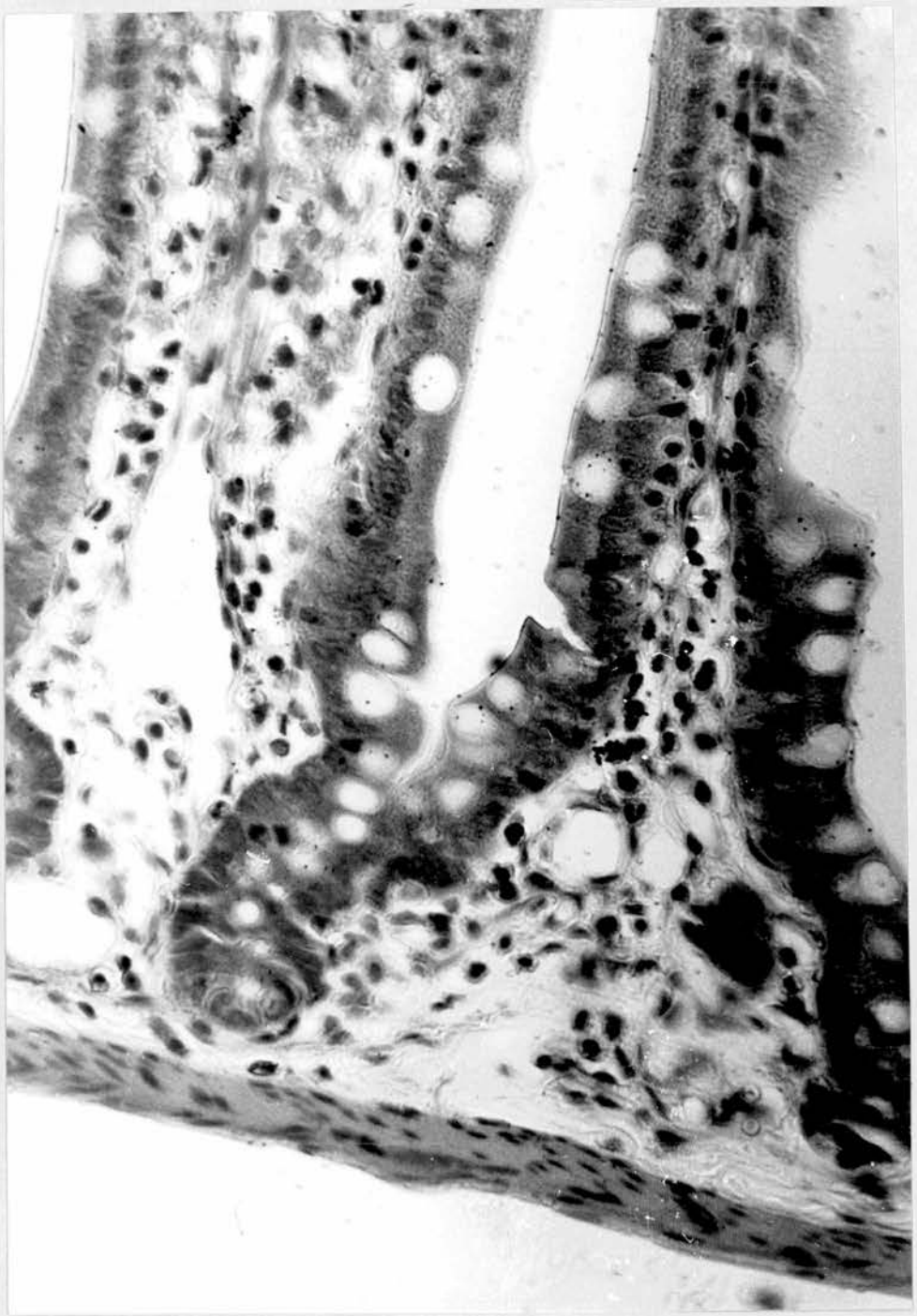
In the small intestine labelled cells were found in the interstitium of the lamina propria as expected. The frequency was greater at the base of the villi compared to the tips and occasional cells were also found in the position of 'theliolymphocytes' between epithelial cells. With ^{3}H or ^{14}C Udr labelled cells those labelled cells seen extravasated in the lamina propria comprised obviously pyroninophilic blast cells and small lymphocytes. ^{3}H Tdr in vitro labelled TDL showed a particular propensity for extravasation in this site as is well established (see Fig 4.12) (Gowans & Knight 1964; Hall, Parry & Smith 1972). However, long lived in vivo ^{3}H Tdr labelled cells were also found in the intestinal lamina propria.

Labelled cells were found frequently in lung at $\frac{1}{2}$ hr and with decreasing frequency at later times. At no time was there evidence of embolization, the cells always being separate and widely distributed throughout the vasculature. Both here and in the liver where labelled cells were found associated with sinusoids and the interstitium of portal tracts, it was very difficult to determine whether many of the cells were in the lumen of vessels or not, even after vascular perfusion with saline and fixative. No evidence of engulfment of labelled cells by Kupffer cells was found.

FIG 4.12

Autoradiograph of a section of the wall of the small intestine of a rat injected 2 hrs previously with 500×10^6 syngeneic TDL labelled with ^{3}H-Tdr (in vitro). Labelled lymphoblasts are present both in the interstitial lamina propria and between epithelial cells (theliolymphocytes). Recipient underwent vascular perfusion at the time of sampling; the small vessels are therefore widely dilated.

Mag x 600. Haematoxylin & Eosin.
6 week exposure.



DISCUSSION

The interpretation of the data revealed by distribution of radioactivity depends upon the factors enumerated in the introduction. The results of the experiments involving splenectomy and intra-arterial injection, together with the apparent independence of behaviour of the injected cells, all suggest that following intravenous injection the labelled cells mix in the blood and are all available to the peripheral circulation immediately. The major localization in lung after intra-arterial and intravenous injection originally described by Weisburger et al (1951) was at that time interpreted as a mechanism for the permanent removal of cells. The later 'emigration' was not apparent as the tissues were only examined at short intervals after injection. The most reasonable interpretation is that the lymphocytes take a relatively long time to traverse the lung capillaries but that they are mobile and not 'sequestered' as suggested by others (Gowans & Knight 1964; Hall Scollay & Smith 1976).

The similarity between the results reported relating to secondary lymphoid tissue and those of various other workers supports the contention that the cells are behaving 'normally'. Thus the major pathways through lymph nodes and spleen were confirmed for small lymphocytes (Gowans & Knight 1964; Goldschneider & McGregor 1968a), as were those through lymph nodes, spleen and small intestine for the lymphoblasts in thoracic duct lymph (Gowans & Knight 1964; Griscelli, Vassali & McCluskey 1969; Hall, Parry & Smith 1972).

In addition to these well defined migratory routes the present data suggest that a further major pathway is through the parenchyma of the bone marrow. As early as 1951, Farr reported the presence of many fluorescently labelled isologous lymph node cells in the bone marrow of rabbits at early intervals after iv injection with a disappearance from this site by twelve hours. These

results were interpreted at that time as being suggestive of transformation into other cell types rather than re-migration which was not considered. Shorter & Bollman (1960), using ^{51}Cr labelled lymphocytes in rats, reported maximum bone marrow localization of 4% of the injected dose, but the size of the sample was not published. The presence of some interchange of long-lived lymphocytes between blood and bone marrow has been suggested by the presence in bone marrow suspensions of cells with similar functions - eg GVH activity (Yoshida & Somond 1971) and SRBC 'helper' cells - to those of mature T cells which could be removed by prolonged thoracic duct drainage (Howard & Scott 1972). This interchange has always been assumed to be relatively small, but it seems that a substantial flux of such cells occurs. The estimate of roughly a quarter of the injected cells being temporarily localized in the bone marrow was made assuming that the total bone marrow organ was contained in bones totalling 5% of body weight in similar proportion to the tibiae and femora. The total skeleton weighs slightly more than this but some bones contain very little marrow. The large size of this flux has not been appreciated for two main reasons. Firstly, the tissue is diffusely spread throughout the skeleton and is perhaps more difficult to examine than other tissues and, secondly, the rapid flux means that the maximal localization is soon after cell transfer. In the equilibrated state the proportion of recirculating cells within the marrow lymphocyte population is very small, as shown by the very high labelling index in this population after only a few days ^{3}H Tdr administration (Everett et al 1964). Chapter 6 will discuss experiments related to the importance of this particular migratory pathway.

The evidence relating to the efficiency of perfusion wash-out of the vasculature suggested that a maximum of ten percent of the activity in most tissues could be accounted for by remaining intravascular label. This low value of intravascular contamination together with the histological evidence of labelled cells in the interstitium of tissues

and particularly in the subcapsular sinus of lymph nodes, even at early intervals after in injection, support the hypothesis that the bulk of the total activity measured in each tissue is related to the extravascular content of labelled cells.

If this is the case estimations of the proportional distribution after equilibrium has been achieved within the recirculating pool, will provide some measure of the relative size of the many compartments of the total pool, remembering always that the degree of accuracy will be limited by a tendency to over-estimation because of some intravascular label and also a tendency to under-estimation following elution of label.

With a knowledge of the size of the total pool, these proportions within various compartments can be converted to actual numbers of lymphocytes. Furthermore, if the modal transit time through a compartment can also be estimated, then tissue fluxes in numerical terms can also be calculated. Allowing that equilibration is virtually achieved by 24 hrs, by which time some ten percent of ^{51}Cr has been secreted in the urine, the proportions at this time interval for the passaged ^{51}Cr TDL population can be used to estimate numbers of accredited recirculating cells in each compartment. The total numbers in the whole pool were estimated using the assumption of Gowans (1959) that the total output in the thoracic duct lymph over the period of about four days, by which time low plateau levels of cells were draining reflected the number of readily mobilized cells of the recirculating pool. In the rat strains used for these experiments average values of $2 - 2.5 \times 10^9$ cells were collected over a period of four days continuous drainage.

Another source of error when estimating total organ proportions is the variation in weight of each tissue. The degree of error introduced is likely to be greater for organs with high concentrations of label such as lymph

nodes, especially as multiple small organs are difficult to dissect out totally with certainty. In large diffuse organs such as skin or muscle, because of the low label concentrations, relatively large errors in the weight estimation will make relatively little difference to the total organ content of labelled cells.

With the use of the 24 hr ^{51}Cr passaged cell data, the standard organ weights in a 200g rat and the value of 2.5 x 10^9 for the total pool content, values for lymphocyte content of tissues were calculated and are shown in Table 4.12.

TABLE 4.12

Tissue	Approx.content of recirc.lymphocytes x 10^6/whole organ	Approx.content of recirc. lympho-cytes x 10^6/gm/ tissue
Spleen	427.0	1070.0
Sup.lymph nodes	650.0	1300.0
Mesenteric node	160.0	1060.0
Small intestine	38.5	7.7
Thymus	.65	2.2
Bone marrow	60.0	6.0
Blood leucocytes	42.5	2.8
Lung	55.0	46.0
Liver	232.0	23.2
Kidney	13.0	8.0
Ovary	.075	.75
Uterus	.10	.25
Muscle	9.5	.09
Salivary gland	.43	1.2
Brain	.05	.03
Skin	4.5	.15
TOTAL	1683	

These figures can only be regarded as approximate and it

is noted that the total accountability is only about two thirds of the estimated total pool. The majority of this shortfall is likely to be due to inaccuracies in lymph node weight estimates. However, artefactual results may obtain for tissues such as liver and kidney, as these are known to be organs involved in disposal of label associated with dead cells. The same may also be true in salivary gland, for no labelled cells were found in this organ in histological sections and it is a secretory organ that might be expected to act in the excretion of cell free label. The values relating to the lung are after perfusion and were measured in peripheral pulmonary tissue. The degree of extravascular label is unknown, but a major proportion in this situation may still be intravascular. The results suggest that at least half of the total cells in the circulation are in the pulmonary vasculature and therefore might escape being counted in systemic blood samples. Put another way, the total blood leucocyte count measured by systemic blood samples may largely underestimate the true total intravascular complement of such cells.

The results for spleen and lymph nodes compare closely with those of Ford & Gowans (1969). Estimates of tissue fluxes from these results require also an estimate of the modal transit time. Deriving values for this parameter from the shape of the distribution curves (the time of peak localization and the rate of subsequent decline) and assuming uniformity in the behaviour of the cells within the injected population tissue flux can be calculated using the formula:

$$\frac{\text{No. of lymphocytes/gm}}{\text{Transit time in hrs}}$$

Using direct evidence relating to the transit time in spleen of 5-6 hrs (Ford 1969a) and lymph nodes 18 hrs (Ford & Simmonds 1972) as approximate calibrations for the other curves, transit times are suggested in Table 4.13.

TABLE 4.13

Tissue	Est.Transit Time Hrs (approx)	Lymphocyte Flux /gm/hr	Lymphocyte Flux /Organ/hr
Spleen	4-6	214×10^6	86×10^6
Sup.lymph nodes	18	72×10^6	36×10^6
Small intestine	?12	$.64 \times 10^6$	3.2×10^6
Thymus	18	$.12 \times 10^6$	$.04 \times 10^6$
Bone marrow	3-5	1.2×10^6	12×10^6
Blood leucocytes	.3	9.3×10^6	140×10^6
Lung	?? mins	-	
Liver	?	-	
Kidney	?	-	
Ovary	1-3	$.25 \times 10^6$	$.025 \times 10^6$
Uterus	1-3	$.083 \times 10^6$	$.033 \times 10^6$
Muscle	1-3	$.03 \times 10^6$	3.0×10^6
Salivary gland	1-3	$.40 \times 10^6$	$.12 \times 10^6$
Brain	1-3	$.01 \times 10^6$	$.015 \times 10^6$
Skin	1-3	$.05 \times 10^6$	1.5×10^6

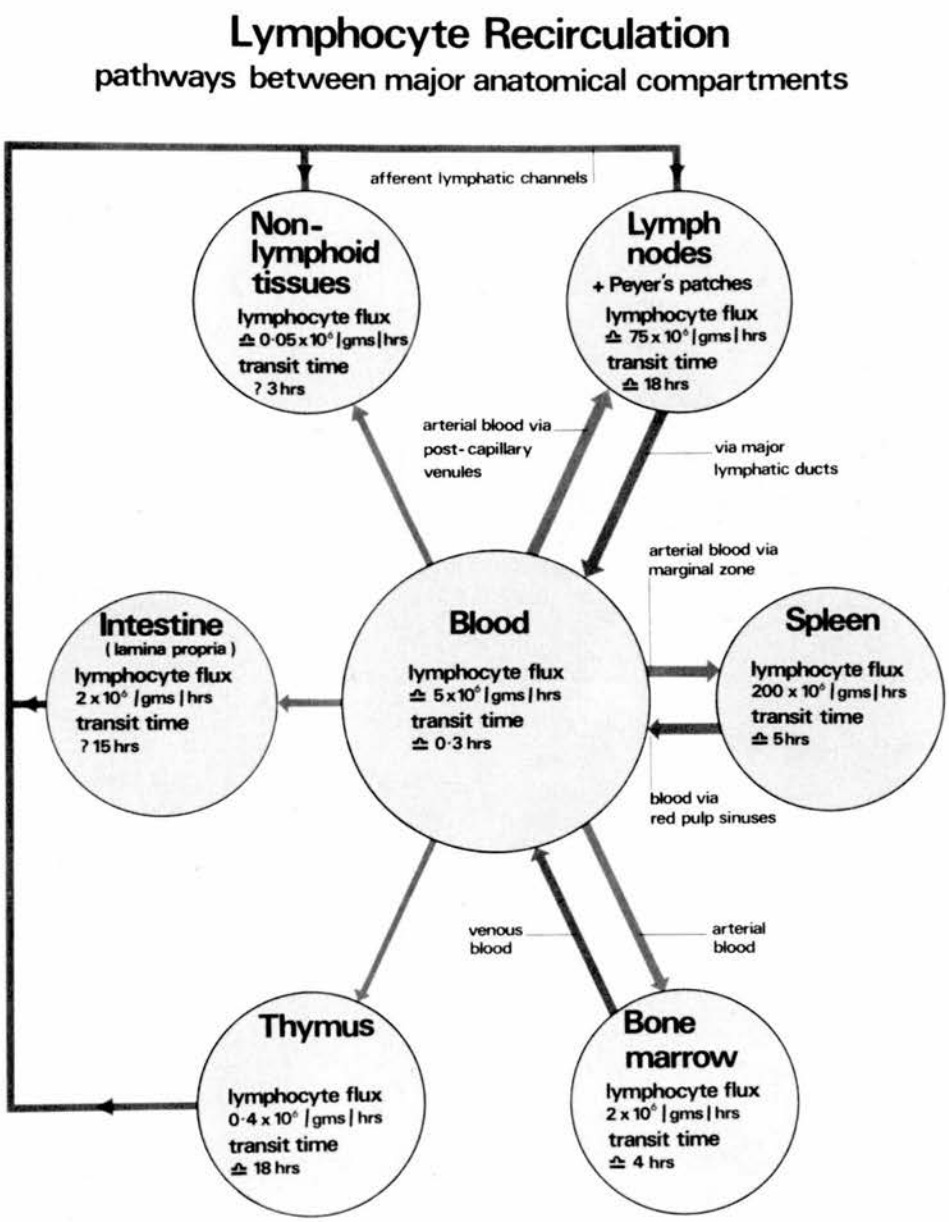
Apart from the histological evidence of labelled cells in lymph node subcapsular sinuses of draining lymph nodes within one hour, support for the contention that modal transit times through non-lymphoid tissues are more rapid than through lymph nodes, comes from experiments in the sheep, in which ^{51}Cr labelled efferent lymph cells were injected iv and collected from both afferent and efferent lymph duct fistulae. In these experiments activity was consistently found in afferent lymph within the first hour and the activity curve in afferent lymph lead that in efferent lymph by several hours (Hall, Scollay & Smith 1976). A transit time of approximately ten hours was suggested and if this was so the present fluxes for the rat could be reduced by a factor of about three. The presentation of the results in this report as specific activities precludes detailed quantitation of the kinetic data.

The results of experiments of Smith et al (1970a) which examined cell outputs in afferent lymph from several tissues in the sheep give precise quantitation of cell numbers but no estimate of the weight of tissue drained was reported. For discreet organs, where the total lymph output can be reasonably assumed if organ weights are also estimated then fluxes can be computed. Hourly outputs in afferent lymph of 2.0×10^6 from the kidney, 5.0×10^6 for the testis and 75×10^6 for the liver, were quoted (Smith Pederson & Morris 1970). If weights for these organs of 100gm, 250gm and 1000gm are assumed, then flux values of .02, .02 and $.075 \times 10^6$/gm/hr are suggested. These values are of the same order of magnitude as those calculated for non-lymphoid tissues in the present experiments. Fig 4.13 shows a schematic view of the compartments of the re-circulating lymphocyte pool with possible rates of flux.

The finding of a substantial afferent supply of small lymphocytes to hepatic node from the liver in the sheep which amounted to about half of the efferent output of this node, provides evidence for a substantial migration pathway through liver. This is also suggested by the early increased concentration of label in this node compared to others and the very frequent finding of labelled cells in subcapsular sinuses in this node as has also been described for ^{32}P and ^{3}H-Tdr-labelled lymphocytes (Fichtelius & Groth 1963). However because of the lack of fluctuation in tissue levels in the liver, a modal transit time cannot be suggested.

A very indirect method of estimation of this hepatic flux can be made by assuming half of the activity in the hepatic node to be the result of this afferent flow. The hepatic node weighing approximately 0.01gm would therefore contain some 15×10^6 cells as a result of afferent flow at equilibrium. If the hepatic weight and transit time were 10g and 3 hrs respectively, then a flux of $.5 \times 10^6$/gm/hr through liver is suggested. This is greater than that found in the sheep and little reliance can be placed

FIG 4.13


Lymphocyte Recirculation
pathways between major anatomical compartments
afferent lymphatic channels
Non-lymphoid tissues
lymphocyte flux
≙ 0·05 x 10⁶ | gms | hrs
transit time
? 3 hrs
Lymph nodes
+ Peyer's patches
lymphocyte flux
≙ 75 x 10⁶ | gms | hrs
transit time
≙ 18 hrs
arterial blood via post-capillary venules
via major lymphatic ducts
arterial blood via marginal zone
Intestine
(lamina propria)
lymphocyte flux
2 x 10⁶ | gms | hrs
transit time
? 15 hrs
Blood
lymphocyte flux
≙ 5 x 10⁶ | gms | hrs
transit time
≙ 0·3 hrs
Spleen
lymphocyte flux
200 x 10⁶ | gms | hrs
transit time
≙ 5 hrs
blood via red pulp sinuses
venous blood
arterial blood
Thymus
lymphocyte flux
0·4 x 10⁶ | gms | hrs
transit time
≙ 18 hrs
Bone marrow
lymphocyte flux
2 x 10⁶ | gms | hrs
transit time
≙ 4 hrs

upon it. However, this result suggests that the ten per-cent of the injected dose measured in liver considerably over-estimates the true extravascular content of viable cells.

If these values for lymphocyte flux are reasonable then the total flux through non-lymphoid tissues excluding liver amounts to about 10 x 10^6/hr. This is in agreement with the hypothesis that the majority of lymphocyte traffic into efferent lymph comes directly from the blood in lymph nodes and not via the tissues and afferent lymph, rather than the other way round as originally suggested (Sjovall 1936). The latter theory was also supported by St Marie (1967) who examined arteries and veins at the hilum of lymph nodes in rats quick frozen in liquid nitrogen for their lymphocyte frequency per unit area in tissue sections. He reported an increased frequency in the veins and interpreted this as being due to a net addition of lymphocytes to the blood from the lymph node in direct contradiction to the idea that the blood lymphocytes showed net entry into the node. He further suggested that the autoradiographic evidence in the rat and kinetic data in the sheep could be due to experimental artefact and that the afferent input of lymphocytes into nodes was quantit-atively more important (St Marie 1975). The present results suggest rather that the maximum figure for the afferent input of most nodes is 20% of the total output necessitating a net flux from the blood to the efferent lymph. The clearance factor from blood flowing through a node cannot be estimated accurately without a reliable estimate of the actual blood flow but recent studies using radioactive microsphere embolization to measure blood flow to lymph nodes suggested that about one lymphocyte in five may leave the blood and enter the node (Hay & Hobbs 1977; Herman et al 1976).

The suggested rate of flux through non-lymphoid tissues is of significant size and is presumed to be of functional significance. Whether the supply of antigen reactive

cells is large enough to allow for 'peripheral sensitization' to antigens without the necessity of also invoking antigen transport to lymph nodes remains open to doubt but the efferent arm of the immune response, particularly for cell mediated responses, takes place in the peripheral tissues and must therefore be mediated by cells within the normally migrating population. The important step is the triggering of the inflammatory response which then produces an increased cell emigration. This step is antigen specific and cell mediated and unless antigen is present upon vascular endothelium must rely upon extravasation of 'specific' lymphocytes within the total population.

Thymus

The accumulation of label in this primary lymphoid organ after injection of labelled cells from all three populations studied suggests that the barrier to recirculating cells in this organ is far from complete. Gowans & Knight (1964) reported the failure of ^{3}H-adenosine labelled TDLs to localize in adult thymus, but did find labelled cells in the thymic medulla of new-born rats and these data might be explained as a dose effect as the detection of cells in autoradiographs of this tissue was much easier when large cell inocula were used. Considerable numbers of newly-formed lymph node cells labelled in situ with ^{3}H Tdr by application of the isotope to ischaemic antigen activated lymph nodes were found in the thymic medulla (Werdelin et al 1971). These cells are likely to have been T-blasts and raise the possibility that only lymphoblasts can enter this tissue. The finding of accumulation of ^{51}Cr-passaged TDLs in the thymus suggests that recirculating lymphocytes also have this capability. This raises the question of how much of the activity of cortisone-resistant thymocyte populations is due to recirculating cells that have migrated from blood and how much is due to maturation of thymocytes in situ to a degree of similar functional competence as suggested by

Jacobsson et al (1972). The ability of the thymus to provide an adoptive plaque forming response particularly in primed animals with large booster doses of SRBC iv argues strongly that memory B cells also have the ability to migrate to the thymus. (Benner et al 1975). Plaque forming cells to hen lysozyme protein have also been found in this organ in secondary responses (Hill 1976).

Immunoblasts

Interpretation of the kinetics of distribution of ^{125}I after ^{125}I Udr-labelled blast cell injection must be even more tentative than for ^{51}Cr because of the contaminatory elution and progressively poor total recovery. Difficulties also arise in autoradiographic studies, as the numbers available for labelling are small because only a minority of thoracic duct cells belong to this population. In normal thoracic duct lymph the majority of the lymphoblasts are likely to have been formed in gut-associated lymphoid tissue which provides the major drainage area for this duct below the diaphragm. The cells comprise approximately 70% B-blasts and 30% T-blasts (Crum & McGregor 1976).

The pattern of distribution revealed in these studies agreed closely with that described by others (Gowans & Knight 1964; Hall, Parry & Smith 1972; Griscelli, Vasalli & McCluskey 1969) and suggested that large lymphocytes have a greater propensity than small lymphocytes to leave the circulation in all sites. This increased tendency has been described for inflammatory exudates (Koster & McGregor 1971; Koster et al 1971) and cellular immune tissue inflammatory reactions (Werdelin et al 1972).

Comparative proportional distributions of ^{51}Cr-passaged and ^{125}I Udr-labelled cells in the same recipients reveal $^{125}I/^{51}Cr$ ratios of up to 10.1. Also subtracting the contribution of passaged ^{51}Cr-labelled cells from ^{51}Cr whole TDL and allowing that ^{51}Cr-labelled blasts may have a higher cell specific activity than small lymphocytes

(Eyre, Rosen & Perry 1970), then approximately equal numbers of each cell type are present in most tissues including the small intestine. Support for this idea in experimental autoimmune lesions comes from the experiments of Werdelin & McCluskey (1971) who counted labelled cell frequencies in lesions by autoradiography after injection of equal total numbers of lymphocytes labelled with either ^{3}H-adenosine or ^{3}H Tdr in vitro. Only slightly greater concentrations of adenosine-labelled cells were found despite the very much lower labelling index for the ^{3}H Tdr population.

That this propensity to extravasate is general even though much lower proportions of immunoblasts localize in lymph nodes and spleen in vivo is suggested by the finding of a 2:1 excess of proportional extravasation of ^{3}H Tdr in vitro labelled TDL over ^{14}C Udr labelled TDL in the isolated perfused lymph node preparation (Sedgley & Ford 1976). Thus in vivo the deficit in secondary lymphoid organs must be the result of competition by other tissues, the most important of which is no doubt the small intestine.

The question of specificity of recognition of tissues by lymphocytes particularly in lymph nodes (Griscelli, Vassali & McClusky 1969) may be particularly relevant to lymphoblasts (Rose, Parrot & Bruce 1976 a & b; Hall et al 1977).

The finding of an increased concentration of ^{125}I Udr labelled TDL in the mesenteric node compared to superficial nodes at 2 hrs after injection may be cited as evidence in support of positive recognition of tissue of origin as most blasts in TD lymph will have been produced in this node. Another explanation, however, derives from the autoradiographic evidence of many such labelled cells in the subcapsular sinuses of the mesenteric node even by one hour and an afferent supply of the magnitude of that from the gut lamina propria would not be available from other tissues to superficial nodes. Thus if recognition

does occur it may be at the level of the peripheral tissue vasculature as much as at the level of the lymph node (Rose, Parrot & Bruce 1976).

CONCLUSIONS

The evidence suggested that labelled cells could be used as tracers in the analysis of the recirculating pool as a multicompartmental system. Rough estimates of tissue flux of lymphocytes through non-lymphoid tissues of the order of 10^4 - 10^5/gm/hr were suggested. Such figures were derived for accredited recirculating lymphocytes but may be similar for lymphoblasts.

A major recirculation pathway through the parenchyma of the bone marrow was described which involved a considerable proportion of the injected cells with a time course similar to spleen.

The other primary lymphoid organ, the thumus, was also found to be part of the recirculating pool with a throughput pattern like that of lymph nodes but at very much lower concentrations.

The total flux through non-lymphoid tissues was suggested to be of low volume but rapid and to amount to not more than 20% of the total lymph node throughput.

CHAPTER FIVE

The Migration of Thoracic Duct Lymphocytes to Sites of Inflammation

Introduction

Since the recognition of the central importance of lymphocytes as mediators of immune responses (reviewed by Gowans & McGregor 1965), the accumulation of this cell type in interstitial infiltrations characteristic of many inflammatory lesions has been taken to reflect an immunological component of such responses. In the 'cell-mediated' type of chronic inflamation, in which sensitivity to a particular antigen cannot be adoptively transferred to a naive recipient by serum, the direct interaction of specifically committed lymphocytes with antigen, in the peripheral tissue site of antigen deposition, is presumed to be essential for the triggering of the inflammatory response. Many studies involving the adoptive transfer of hypersensitivity to antigens with lymphocytes support this view but the exact site of cell-antigen interaction and the mechanisms involved in the subsequent accumulation of lymphocytes in inflammatory lesions remain unclear.

A net accumulation of lymphocytes within a tissue could arise if the outflow of lymphocytes was halted or reduced or if the influx of cells was increased. A slowing of the modal transit time of lymphocytes through the tissue would also lead to an apparent accumulation of cells within the interstitial compartment even if the net flux remained constant. The observations of increased lymphocyte outputs in the afferent lymph draining (i) a chronic subcutaneous granuloma in the sheep (Smith, McIntosh & Morris 1970b), (ii) a site of subcutaneous injection of particulate antigen in the rabbit (Kelly R H 1970), (iii) an

allogeneic kidney graft in the sheep (Pederson & Morris 1970 & 1974), argue that there is no reduction in the numbers of lymphocytes leaving the tissues under these circumstances and by inference that extravasation from the blood must be increased. This increased flux of lymphocytes into sites of inflammation has been measured using isotopically labelled lymph node cells usually 24 hrs after iv injection. Asherson & Allwood described the increased localization of ^{51}Cr labelled normal lymph node cells in ears painted with contact sensitizing agents or croton oil and also showed an increased ability of recently divided lymphocytes produced in response to a variety of immunogenic stimuli to localize in both specific and unrelated lesions (Asherson & Allwood 1972). More recently the particular subclass of lymph node cell from immunized mice that shows the highest localization in contact sensitivity lesions has been characterized as a thymus derived lymphoblast (Allwood 1975). Increased accumulation of ^{51}Cr normal lymph node cells in both actively immunized and passively transferred contact sensitivity lesions in mouse ears has also been reported (Sabbadini et al 1974).

Other reports have suggested that immunoblasts have an increased ability to enter inflammatory exudates in the peritoneum (McGregor et al 1971) and in experimental autoimmune diseases (Werdelin 1972). The particular importance of the T-blast in contact sensitivity lesions has been stressed (Rose et al 1976).

This propensity of activated T cells to enter inflammatory lesions may explain at least in part the development of the skin lesion several days after primary sensitization of an animal with an agent such as Picryl Chloride. There are however situations in which delayed type hypersensitivity responses develop when such T blasts would not be expected to be present if it is true that they are relatively short-lived and not part of the true recirculating pool as the evidence suggests (McGregor et al 1971,

Allwood 1975). The tuberculin reaction is of this type and develops within 24-48 hrs after antigen administration in animals immunized many months previously. Memory for this antigen is carried by long-lived small recirculating lymphocytes (Lefford et al 1973). The speed of onset of this reaction probably precludes the activation of memory cells in draining lymph nodes and subsequent initiation of the lesion by migration of lymphoblasts.

Whatever the type of lymphocyte involved the precise site of interaction with antigen and the mechanism whereby this triggers the inflammatory response are unknown. The difficulty encountered in experiments designed to detect any preferential localization of specific antigen reactive cells within inflammatory infiltrates (reviewed by Werdelin 1972) suggests that the extravasated lymphocytes are a random selection of those in the blood with respect to antigen sensitivity although selection with regard to other non-antigen related factors such as size, thymus dependency or tissue of origin may be operative. This non-selection by antigen argues against specific attraction of reactive lymphocytes from the blood, but if the normal flux of cells through the tissue is not great enough to allow emigration of enough specific cells (depending upon their frequency in the blood) to account for the time course of triggering of the lesion, then some mechanism allowing interaction of antigen and lymphocytes within the blood vessels must be considered.

The vascular changes entailed in inflammation almost certainly permit an increased extravasation of lymphocytes. Two mechanisms are possible: release of vasoactive compounds could increase the blood flow locally and provide more lymphocytes to the vasculature which would allow increased extravasation without any alteration in the individual lymphocyte-endothelial cell interaction - the 'clearance factor'. On the other hand a qualitative change in the endothelium could be envisaged which would render lymphocyte adhesion and extravasation more efficient.

These two possibilities are not mutually exclusive and both could operate.

The increase in blood flow to cell mediated lesions, as measured by embolization of radiolabelled microspheres, was considered to be great enough to account for the increase in lymphocyte extravasation (Hay et al 1977). On the other hand the emigration of lymphocytes through venules in the lesions of experimental autoimmune neuritis as studied with phase contrast and the electron microscope had many similarities to that seen in lymph node post-capillary venules which is specific for lymphocytes. Also if no qualitative change in the endothelium occurs then increased lymphocyte extravasation should follow any form of vasodilation - even mild heat - and this does not occur. The observation of morphological change, in some venules in the centre of dense infiltrations of small lymphocytes in muscle in some patients with polymyositis, so that the endothelial cells are cuboidal and pyroninophilic (Ball J pers.comm.) suggests that some 'specialization' can occur but this change is rarely pronounced in most inflammatory lesions.

The experiments to be described were designed to examine the kinetics of localization of normal thoracic duct cells in various inflammatory lesions. These lesions were designed to have different immunological components and to be relatively constant, as far as intensity was concerned over the period of study, to minimize distortion of the results following changes in the lesions themselves. The experiments closely followed the design of those described for normal tissues and the relative contribution of lymphoblasts and small recirculating lymphocytes was again assessed.

In addition, the localization of in vitro trypsin-treated TDL was studied. Such cells have a greatly reduced ability to enter lymph nodes via the post-capillary venules but an unaltered ability to migrate across the splenic marginal sinus endothelium, both of which sites normally support lymphocyte traffic at a high rate and with efficient selection. This altered behaviour is temporary with the cells showing total recovery and normal distribution by twenty four hours (Woodruff & Gesner 1968, Ford et al 1976). This effect can provide a tool to investigate other sites in which a lymph node like lymphocyte-endothelium interaction may operate.

METHODS

1 Cell Labelling

^{51}Cr whole TDL, ^{51}Cr passaged TDL, and ^{125}I Udr in vitro labelled TDL were produced as before.

All recipients underwent whole body perfusion at the time of death at various times after intravenous injection of normal syngeneic labelled TDL.

2 Production of Inflammatory Lesions

a Skin sensitization to chemical agents

Table 5.1 shows the agents used together with the dilutions and diluents.

Rats were sensitized by application of 0.1ml of agent to the clipped abdominal skin. Secondary lesions were elicited by application of 0.1ml of the appropriately diluted agent to a circular area (- 1.5cm in dia.) of clipped skin on the back 12 days later. Similar areas were also painted with 0.1ml of dilute solutions of the other agents at the same time. Thus recipients were sensitized with one agent and challenged with all four compounds, enabling contact hypersensitivity lesions and direct irritant inflammatory lesions to be studied in the same animal.

TABLE 5.1

Agent	Sensitizing Dose	Challenge Dose
1, Chloro 2,4. Dinitrobenzene(a) (DNCB)	5%(c)	0.5%(c)
Picryl Chloride(a) (TNCB)	7%(c)	1.0%(c)
4 Ethoxymethylene-2-Phenyl Oxazolone (OXAZ)(a)	5%(c)	0.5%(c)
Croton Oil(b)	10%(d)	10%(d)

(a) BDH Chemicals Ltd, Poole, Dorset.

(b) Sigma Chemical Co, PO Box 14508, St Louis, USA.

(c) Dissolved in absolute ethyl alcohol.

(d) Dissolved in Di-n-butylphthalate-(a).

Specific contact sensitivity was elicited in primed animals following challenge with a low dose of TNCB, DNCB or OXAZ. Clinically the lesion was an area of erythema with little obvious induration. Histologically a moderate mixed mononuclear cell infiltrate was seen throughout the dermis (see Fig. 5.1) with occasional superficial foci of polymorphonuclear leucocytes. Lymphocytes were present as a minority cell type. Maximum intensity of the inflammation was seen by 24-48 hrs and the lesion remained roughly the same for a period of 48 hrs.

Skin painting with Croton oil produced a mild acute inflammatory reaction with erythema, slight oedema and a mild acute inflammatory cell infiltrate. No evidence of sensitization was found, with a second application producing a lesion of similar severity as the first.

Primary application of challenge doses of TNCB, DNCB or OXAZ, produced no obvious lesion at any time.

b Granuloma

Subcutaneous granulomata were produced by intradermal injection of 0.1ml of an emulsion of equal volumes of Freund's complete adjuvant (Difco) and PBS at several sites on the flanks of potential recipients three weeks before labelled cell injection. Histologically the lesions comprised droplets of oil surrounded by an infiltrate of macrophages, fibroblasts and relatively few lymphocytes (see Fig 5.2).

c Tumour

A mammary carcinoma, which arose spontaneously in a rat of the DA strain, was passaged by serial subcutaneous implantation of small fragments of tumour tissue in females of either DA or PVG/c x DA F_1 hybrid strain. Potential recipients of labelled

FIG 5.1

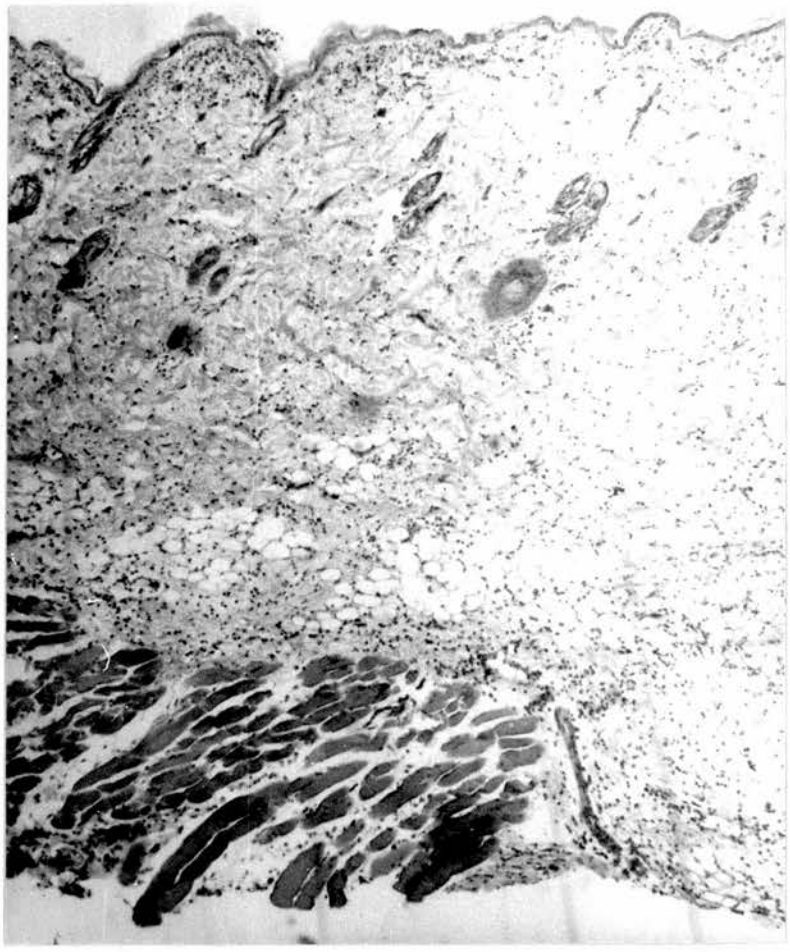
Histological section of skin from a rat sensitized 12 days previously with DNCB, showing the edge of an area challenged by painting with DNCB 48 hrs previously. A diffuse mononuclear cell infiltrate which includes lymphocytes is present in the deep dermal connective tissue and muscle; in addition there are superficial foci of acute inflammatory exudes in which polymorphonuclear leucocytes predominate.

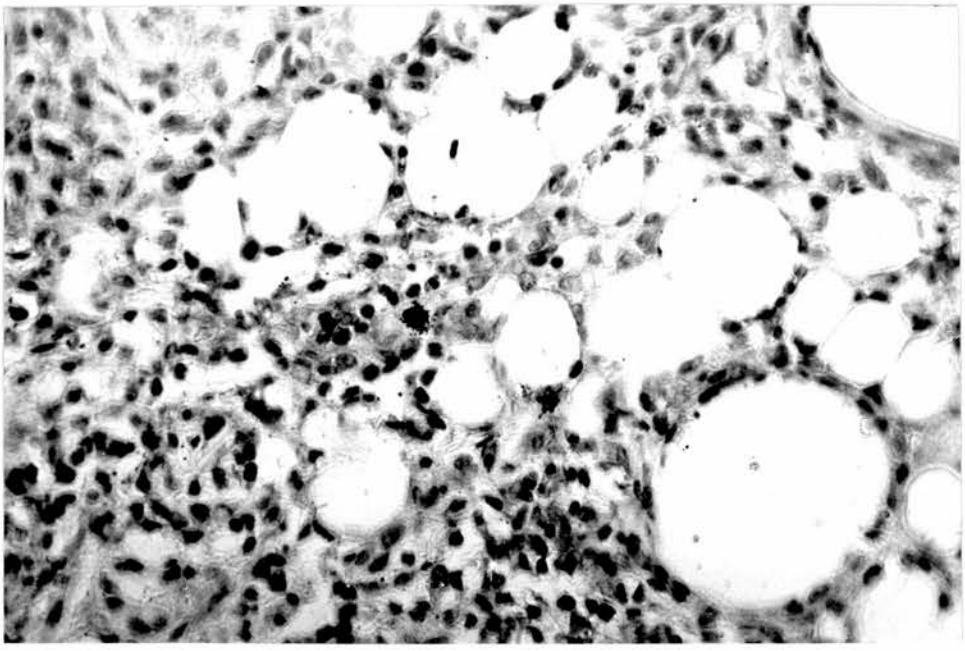
Mag. x 70. Haematoxylin + Eosin.

FIG 5.2

Autoradiograph of a granuloma in the deep dermis of a rat injected i.d. with an emulsion of Freund's complete adjuvant and PBS 21 days previously and iv 2 hrs previously with 300×10^6 syngeneic TDL labelled with ^{3}H-Tdr (in vitro). Large globules of lipid are surrounded by macrophages, lymphocytes and fibroblasts. Two labelled lymphoblasts can be seen in the interstitial cellular infiltrate.

Mag. x 450. Methyl Green + Pyronin.
6 weeks exposure.





cells received up to four subcutaneous fragments at separate sites on the flanks and the tumours were allowed to grow to approximately one centimetre diameter nodules. At this size the tumour comprised solid masses of malignant epithelial cells with relatively little fibrous stroma or interstitial infiltrate but a well defined capsule. The tumours were well vascularized and only showed necrosis + haemorrhage when larger than 1.5 diam. No metastatic spread was encountered.

d Trypsin Treatment of Lymphocytes

Radiolabelled TDL were washed in PBS and resuspended at 10^8/ml in protein free PBS to which was added 0.02mg/ml final concentration of Trypsin (Difco). The cells were incubated at 37°C for 10 mins, after which FCS was added to a final concentration of 10% to inhibit further trypsinization. The cells were then filtered through cotton gauze and washed twice before resuspension for injection. Control cells were treated in parallel but incubated in protein-free PBS without trypsin.

Very few cells were lost with this procedure and the viability of the final suspension as assessed by Trypan Blue dye exclusion was always greater than 95% as for the control cells.

e Autoimmune Inflammations

This type of inflammatory lesion has the potential advantages of using normal tissue as antigen and therefore avoiding traumatic administration of antigen at the site of study. This is particularly true in adoptively immunized animals in which the time course of the lesion is also under precise control.

However, several attempts to elicit this type of reaction in various strains of rat all failed to produce significant or reproducible clinical disease

or histological lesions. Antigens used were Guinea-pig Myelin Basic Protein, and crude saline extracts of rat brain, kidney, muscle, testis and epididymis emulsified in varying doses with Freund's Complete Adjuvant. The tubercle bacillus content of this adjuvant was also varied.

Possible reasons for the failure to induce experimental autoimmune encephalitis (EAE) or analogous lesions in other tissues include: (i) Nonsusceptibility of the strains of rat used which were AO (AgB2); PVG/c (AgB5); DA (AgB4); PVG/c x DAF_1 (AgB4 + 5); AS (AgB_1). Most work on EAE has been carried out using Lewis strain rats (AgB1) but no more success was encountered with AS rats of the same AgB type. Variation in susceptibility has been linked to AgB type by Gasser et al (1975) who found both Lewis and DA strain rats to be susceptible and by Hughes et al (1973) who found AS susceptible but PVG/c not. (ii) Inappropriate immunization technique, dose or adjuvant composition. (iii) Failure to use pertussis vaccine as an extra simultaneous adjuvant. This has been reported to increase the frequency and severity of autoimmune diseases induced in this way (Werdelin 1972).

RESULTS

Experiments were carried out in which the localization of ^{51}Cr TDL in skin lesions 24 hrs after iv injection was measured in recipients that had been pre-sensitized with one of the agents listed in Table 5.1 and challenged with all four agents. The results summarized in Table 5.2 show the factor of increase of ^{51}Cr measured in the skin lesions over the concentration measured in normal unpainted skin. It is apparent that the second application of TNCB, OXAZ or DNCB in specifically immunized recipients produced a higher factor of increase than areas challenged with irrelevant agents. Thus antigen specificity of the

TABLE 5.2

THE LOCALIZATION OF ^{51}CR IN SKIN LESIONS 24 HRS AFTER I.V. INJECTION OF ^{51}CR TDLs INTO SYNGENEIC RECIPIENTS 'PERFUSED' AT TIME OF DEATH.

Each recipient had been sensitized to one agent some days previously and challenged with all four agents at the time of labelled cell injection

* Indicates lesion due to second application of agent.

EXPT. NO.		RAT STRAIN	DAYS AFTER IMMUNIZATION	% INJ. ACT PER GM NORMAL SKIN	FACTOR OF INCREASE OVER NORMAL SKIN IN LESIONS			
					TNCB	OXAZ	DNCB	CROTC
57.1	A	AO	7	.0120	1.9*	0.8	1.5	2.5
	B			.0248	0.7	1.9*	0.9	1.5
	C			.0150	1.6	1.3	7.0*	3.9
	D			.0193	1.8	1.7	2.1	3.2*
57.2	A		8	.0120	1.6*	1.0	1.6	2.5
	B			.0178	1.4	2.2*	1.6	2.2
	C			.0116	2.0	1.3	4.9*	2.8
	D			.0108	1.8	1.2	2.2	3.2*
59.1	A	DA x PVG/c F_1	11	.0087	15.9*	2.5	1.7	12.6
	B			.0097	3.1	11.8*	1.8	7.1
	C			.0138	2.9	1.1	11.7*	7.7
	D			.0142	1.9	1.0	1.9	8.9*
59.2	A		12	.0128	17.7*	0.6	1.6	9.5
	B			.0109	4.4	22.8*	2.2	15.9
	C			.0122	2.7	2.8	7.8*	11.8
	D			.0118	3.6	0.9	2.1	12.4*

sensitivity lesions was confirmed. The factor of increase in the area of skin painted with Croton oil was however as large on primary as secondary application, suggesting that there is little or no element of hypersensitivity in this lesion.

Table 5.3 confirms these results and shows the pooled results of several experiments in which the 24 hr localization of ^{51}Cr TDL was measured in inflammatory lesions including those following primary and secondary application of skin paints and also the subcutaneous granuloma and tumour.

It is therefore reasonable to assume some immunological component in the specific recall contact sensitivity lesion while the lesions following primary application of challenge doses of these sensitizing agents provide controls for the direct irritation produced by skin painting; the Croton oil lesion also reflects non-specific irritation but of greater magnitude.

The kinetics of localization of radioactivity following injection of labelled TDL was studied in recipients that had been sensitized to DNCB twelve days previously and challenged with all four agents 24 hrs previously. This early challenge was to allow the lesions time to develop fully before cell injection.

Table 5.4 shows the results of experiments in which ^{51}Cr was measured at $\frac{1}{2}$, 2, and 24 hrs after ^{51}Cr whole TDLs were injected iv. The activity in normal skin followed the pattern described in the previous chapter with an early peak at $\frac{1}{2}$ hr. Concentrations then fall and the 2 hr value was often fractionally below that measured at 24 hrs. A very similar pattern was seen in skin painted with sensitizing agents in non-sensitized recipients (TNCB and OXAZ). In specific recall contact sensitivity lesions ($DNCB^1$) a considerably increased concentration of ^{51}Cr was already present by $\frac{1}{2}$ hr after injection and this concentration usually showed a slight fall between $\frac{1}{2}$ and 2 hrs and then

TABLE 5.3

Localization of ^{51}Cr in Inflammatory Lesions

Arithmetic mean percent inj.activity/gm tissue ± SE in recipients of ^{51}Cr TDL perfused 24 hrs after iv injection.

[1] indicates recipients had previously been immunized with the same agent.

Tissue	No.of samples	% Inj.activity ± SE
Skin TNCB	36	.0265 ± .0022
Skin TNCB[1]	6	.0984 ± .0327
Skin OXAZ	38	.0154 ± .0015
Skin OXAZ[1]	4	.1122 ± .0485
Skin DNCB	18	.0266 ± .0019
Skin DNCB[1]	30	.0688 ± .0069
Skin Croton	40	.0819 ± .0072
Skin Croton[1]	4	.0922 ± .0263
Normal skin	50	.0130 ± .0005
Granuloma	12	.2364 ± .0231
Tumour	4	.0736 ± .0078

TABLE 5.4

The Kinetics of Localization of ^{51}Cr in Skin Lesions in recipients 'perfused' at intervals after iv injection of ^{51}Cr whole TDLs.

Arithmetic mean percent inj.activity/gm of tissue ± SE (pooled data from experiments in which each time period was studied with the same cell suspension).

Tissue	½ Hr	2 Hr	24 Hr
Skin TNCB	.0200 ± .003 (8)[2]	.0134 ± .003 (9)	.0151 ± .001 (9)
OXAZ	.0200 ± .003 (8)	.0153 ± .002 (10)	.0167 ± .003 (10)
DNCB[1]	.0416 ± .003 (20)	.0360 ± .005 (11)	.0689 ± .011 (11)
Croton	.0342 ± .004 (11)	.0281 ± .003 (13)	.0391 ± .008 (11)
Normal	.0272 ± .003 (13)	.0122 ± .001 (10)	.0137 ± .001 (10)

[1] indicates recipients were specifically sensitized with this agent 12 days previously.

()[2] indicates no. of samples.

some late accumulation by 24 hrs. The increased concentration over normal skin in the Croton oil lesion was also present by ½ hr and again a slight trough was seen at 2 hrs with a little further increase by 24 hrs.

In one experiment (Table 5.5) in which the late time course of ^{51}Cr TDL localization was studied in animals which were challenged three hrs after labelled cell injection, when the blood concentration would have been minimal, the usual pattern of accumulation was present by 24 hrs and little difference was seen between these values and those measured at 48 or 72 hrs, by which time some fluctuation in the intensity of the inflammation may have influenced the results.

In order to assess the contribution of the minor population of lymphoblasts in TDL the distribution of ^{125}I was studied in recipients of TDL labelled in vitro with ^{125}I Udr. With this population a somewhat different kinetic distribution pattern emerged. Table 5.6 summarizes data from experiments similar to those in Table 5.4 but using ^{125}I Udr labelled TDL. The concentrations of ^{125}I in normal skin were highest at ½ hr with a subsequent progressive fall. As in the previous experiments the absolute proportion of ^{125}I found in normal skin was higher than with ^{51}Cr whole TDL at all times. In contrast to this latter situation the areas, painted with challenge doses of sensitizing agents to which the recipients had not been previously exposed, showed an increase in localization of ^{125}I between ½ and 2 hrs, and then a marked decrease to 24 hr values. Specific recall lesions showed a similar increase at these early periods but showed less decrease by 24 hrs so that by this late time there was an obvious difference between 'primary' and 'secondary' lesion concentrations. Croton oil irritant lesions showed considerable increase over normal skin at ½ hr with an increase to 2 hrs and if anything still further increases by 24 hrs.

The difference in the pattern of distribution of ^{125}I

TABLE 5.5

Late Time Course of Localization of ^{51}Cr in Skin Lesions

Recipients 'perfused' 24,48 or 72 hrs after iv injection of ^{51}Cr whole TDLs and challenged with all four agents 3 hrs after labelled cell injection.

	No. of Samples	Mean % Inj.Act/gm tissue ± SE		
		24 hr	48 hr	72 hr
Skin TNCB	2	.014 ± .009	.014 ± .015	.015 ± .003
TNCB[1]	1	.040	.045	.049
OXAZ	2	.008 ± .001	.015 ± .006	.008 ± .002
OXAZ[1]	1	.010	.015	.013
DNCB	2	.029 ± .007	.018 ± .002	.023 ± .02
DNCB[1]	1	.113	.110	.117
Croton	3	.157 ± .029	.102 ± .024	.095 ± .018
Normal	3	.011 ± .004	.010 ± .004	.010 ± .003

[1] indicates specifically immunized recipient.

TABLE 5.6

The Kinetics of Localization of ^{125}I in Skin Lesions in recipients 'perfused' at intervals after iv injection of ^{125}I Udr-in vitro labelled TDLs.

Arithmetic mean percent inj.activity/gm of tissue ± SE (pooled data from experiments in which each time period was studied with the same cell suspension.

Tissue	½ Hr	2 Hr	24 Hr
Skin TNCB	.282 ± .060	.333 ± .055 (5)	.121 ± .009
OXAZ	.321 ± .017	.424 ± .025 (5)	.154 ± .012
DNCB[1]	.392 ± .039	.426 ± .074 (5)	.296 ± .078
Croton	.495 ± .055	.541 ± .054	.641 ± .337
Normal	.175 ± .0.5	.111 ± .006	.073 ± .004

n = 4 except where stated.

[1] indicates specific immunization of recipient with this agent.

compared with ^{51}Cr suggests that the activities measured with the latter isotope associated with the whole population of TDL cannot be due only to the lymphoblasts. Two approaches to the estimation of localization of small recirculating lymphocytes in skin lesions were made. 'Passaged' ^{51}Cr labelled TDLs and long-lived in vivo ^{3}H Tdr labelled TDL were used but the production of either of these populations in sufficient quantities to allow adequate 'counts' to accumulate in non-lymphoid tissues in multiple recipients within an experiment was difficult.

Table 5.7 shows the pooled results of two experiments in which recipients bearing skin lesions were 'perfused' and sampled at each time period after passaged ^{51}Cr TDL injection. Again all areas of skin showed peak values of ^{51}Cr at half an hour after iv injection of passaged ^{51}Cr TDL and at this time no difference was seen between painted + non-painted areas. The fall by two hours and 24 hrs was greater than for ^{51}Cr whole TDL and was only slightly less steep for the specific recall sensitivity lesion and the Croton oil lesion which did both show slight evidence of increased concentration over normal skin at 24 hrs.

Table 5.8 shows the factor of increase in concentration of ^{3}H in skin lesions over unpainted skin in recipients perfused 24 hrs after iv injection of long-lived ^{3}H Tdr in vivo labelled TDL. There was an obvious increased concentration over normal skin in the specific recall contact sensitivity lesion to DNCB[1] but no increase in other areas including that painted with Croton oil. The absolute concentration of tritium in skin may be artefactually high because of the likelihood of contamination with non-cell-associated isotope.

Another way of comparing the distribution of the lymphoblasts in TDL with that of the whole population is to examine the ratio between the proportions of each population in each tissue. This comparison is perhaps most

TABLE 5.7

The Kinetics of Localization of ^{51}Cr in Skin Lesions in recipients 'perfused' at intervals after iv injection of 'passaged ^{51}Cr TDLs'.

Tissue	No.	Arithmetic mean percent inj.act/gm tissue ± SE ½ Hr	2 Hr	24 Hr
Skin TNCB	2	.0293±.0036	.0088±.0017	.0055±.0016
OXAZ	2	.0284±.0019	.0069±.0011	.0069±.0025
DNCB[1]	2	.0295±.0014	.0125±.0025	.0090±.0011
Croton	3	.0226±.0021	.0074±.0008	.0095±.0032
Normal	3	.0319±.0032	.0089±.0014	.0067±.0028

No. = number of samples

[1] indicates specific immunization with this agent.

TABLE 5.8

Localization of Tritium in Skin Lesions 24 hrs after iv Injection of Long-lived ^{3}H Tdr in vivo Labelled TDLs

Both recipients were 'perfused' at the time of death.

Results expressed as % inj.activity/gm of normal skin and a factor of increase over this value in the lesions.

Expt No.	Rat Strain	Days after imm.	% inj.act/gm normal skin	FACTOR OF INCREASE OVER NORMAL SKIN			
				TNCB	OXAZ	DNCB[1]	CROTON
58.1 A	AO	12	.041	0.8	1.0	9.2	1.0
B	AO	12	.031		0.8	9.4	1.4

meaningful when carried out in the same recipient of a mixture of labelled cell types but comparisons between groups of results can also be made if the scatter of these values is not too great. In recipients of both ^{51}Cr and ^{125}I labelled populations at 24 hrs after iv injection the $^{125}I/^{51}Cr$ ratio in the DNCB[1] recall lesion was similar to that in normal skin. A similar result is shown in Table 5.9 in which the ratio of percent ^{125}I/gm/percent ^{51}Cr/gm is calculated from the pooled data in Tables 5.4 and 5.6.

The similarity between the ratios of $^{125}I/^{51}Cr$ in the 'recall' lesion and normal skin suggest that there is no marked change in the rate of extravasation of the lymphoblast subpopulation into this lesion but the increased ratios in the irritant lesions supports the idea that newly formed cells may have a particularly increased propensity to leave the circulation under these circumstances.

The effect of trypsin treatment of lymphocytes on their ability to localize in inflamed skin was studied using ^{51}Cr whole TDL. Table 5.10 summarizes the results of three experiments in which the kinetics of localization of ^{51}Cr associated with normal or trypsin-treated TDL were compared. The results are expressed as the mean percent injected activity/gm of tissue for each population in separate groups of recipients. The localization of lymphocytes in normal skin was clearly not impaired by trypsin; radioactivity associated with trypsin-treated TDL found in the skin at $\frac{1}{2}$ and 2 hrs after injection was equal or slightly greater than radioactivity associated with untreated cells, possibly reflecting the marginally higher numbers in the blood.

The spleen and liver showed no decrease in isotope localization following trypsinization of TDL at early times especially when compared to the blood levels confirming the observations of others (Woodruff & Gesner 1968, Ford et al 1976). A similar picture is seen in most non-lymphoid

TABLE 5.9

Ratio of $^{125}I/^{51}Cr$ in Tissues Derived from Tables 5.4 and 5.6

(Mean % inj.^{125}I/gm/mean % inj.^{51}Cr/gm) associated with respectively ^{125}I Udr in vitro labelled TD lymphoblasts and ^{51}Cr in vitro labelled whole TDL.

	½ Hr	2 Hr	24 Hr
Skin TNCB	14.1	24.8	8.0
OXAZ	16.1	27.7	9.2
DNCB[1]	9.4	11.8	4.3
CROTON	14.5	19.3	16.4
NORMAL	6.5	9.1	5.3

TABLE 5.10

The Effect of Trypsin Treatment on the Localization of ^{51}Cr in Skin Lesions and Tissues at Intervals after iv Injection of ^{51}Cr Whole TDLs

Arithmetic mean % inj.act./gm Tissue ± SE. (n - No. of samples = 4 except where stated).

	½ Hr		2 Hr		24 Hr	
	CONT	TRYP	CONT	TRYP	CONT	TRYP
Skin TNCB	.0201± .001	.0195± .003	.0116± .002	.0114± .001	.0119± .002	.0117± .002
OXAZ	.0204± .005	.0225± .002	.0123± .002	.0136± .002	.0139± .002	.0132± .001
DNCB[1]	.0438± .003(1)	.0341± .002	.0323± .011	.0202± .003	.0532± .015	.0410± .009
CROTON	.0284± .005	.0268± .005	.0179± .004	.0184± .004	.0205± .003	.0200± .005
NORMAL	.0294± .004(2)	.0316± .004	.0128± .003	.0131± .002	.0140± .001	.0113± .001
BLOOD LEUCOCYTES /ml	.404 ± .09(2)	.508 ± .08	.066 ± .01	.147 ± .02	.140 ± .01	.140 ± .01

TABLE 5.10 contd

Tissue	½ HR		2 HR		24 HR	
	CONT	TRYP	CONT	TRYP	CONT	TRYP
Cervical lymph nodes	11.3 ± 3.1(2)	.108 ± .01	16.8 ± 4.4(1)	.353 ± .08	58.2 ± 8.7	51.6 ± 7.6
Spleen	82.1 ± 5.7(2)	104.7 ± 7.9	94.6 ± 7.9	139.6 ± 8.2	50.8 ± 2.7	52.5 ± 2.1
Muscle	.013 ± .002	.019 ± .002	.006 ± .001	.008 ± .002	.006 ± .001	.004 ± .00
Liver	2.1 ± .3(2)	2.8 ± .5	1.2 ± .2	1.5 ± .1	1.2 ± .1	1.3 ± .1
Kidney	.51 ± .05(2)	.71 ± .07	.40 ± .02	.47 ± .04	.47 ± .02	.51 ± .01
Ovary	.12 ± .03	.31 ± .01	.11 ± .03	.15 ± .04	.09 ± .02	.06 ± .01
Uterus	.025 ± .002	.026 ± .004	.020 ± .003	.015 ± .003	.018 ± .004	.025 ± .00
Brain	.011 ± .003	.004 ± .001	.007 ± .002	.005 ± .001	.006 ± .002	.009 ± .00
Lung	15.2 ± 3.6(2)	17.7 ± 3.3	1.8 ± .1	2.7 ± .3	2.3 ± .2	1.7 ± .2
Small intestine	.665 ± .04(2)	.117 ± .02	.737 ± .111	.232 ± .05	.821 ± .07	.394 ± .06
Thymus	.052 ± .01(2)	.025 ± .002	.048 ± .01	.034 ± .001	.202 ± .06	.130 ± .04
Bone (marrow)	3.5 ± .35(2)	2.4 ± .33	3.7 ± .25	2.5 ± .05	.88 ± .11	.69 ± .12

(1) n = 16 - difference between control & trypsin significant; $P < .02$ - Mann-Whitney U test.

(2) n = 7 - for control and trypsin groups.

tissues suggesting that migration of lymphocytes into non-lymphoid tissues in general may be trypsin-resistant. By contrast the localization of ^{51}Cr in lymph nodes was reduced to 1-2% by trypsin at $\frac{1}{2}$ and 2 hrs with complete recovery by 24 hrs.

The most striking result was that trypsin treatment did impair the capacity of ^{51}Cr TDL to migrate into established recall contact sensitivity lesions. Although at $\frac{1}{2}$ and 2 hrs in these experiments the localization of ^{51}Cr in DNCB[1] lesions with control cells was only about 2.5 times greater than in normal skin this increase was highly significant ($P \leq .02$) and consistent. With the trypsin-treated populations little or no increase over the normal skin values occurred at these early times and the difference in absolute values in the lesion due to control and trypsin-treated cells was also significant ($P \leq .02$). No inhibition of migration of trypsin-treated cells was measured in the sites of primary application of sensitizing agents and this was probably also true for the Croton oil lesion but the increases in this latter lesion with control cells were very small in this particular series of experiments.

This inhibition of migration of trypsin-treated ^{51}Cr TDL into the cell mediated immune lesion is most strikingly seen if the increase in activity that could be attributed to the immune inflammatory component of the lesion is taken to be the excess localization over normal skin values. Table 5.11 shows the concentration of ^{51}Cr/gm of inflamed skin minus the concentration of ^{51}Cr/gm of normal skin for each animal. The mean of these differences $\pm$ SE are shown. Again a clear effect is seen in the DNCB[1] lesion particularly at $\frac{1}{2}$ hr but still obvious at 2 hrs but no differences between the localization of control or trypsinized cells in the Croton oil or other irritant lesions are seen.

Examination of another chronic inflammatory lesion similar

TABLE 5.11

Excess Localization of ^{51}Cr over normal skin in inflamed skin after i v injection of normal or trypsin-treated ^{51}Cr TDLs

Arithmetic mean % inj.act/g - background $\pm$ S E.

Values derived from same recipients as in Table 5.10

Tissue	½ Hr		2 Hr		24Hr	
	Control	Trypsin	Control	Trypsin	Control	Trypsin
DNCB'	.0169 $\pm$ .018 (n = 16)	−.0025 $\pm$ .0015	.0193 $\pm$ .001 (n = 4)	.0071 $\pm$ .001	.0383 $\pm$ .015 (n = 4)	.0298 $\pm$ .009
Croton	.0023 $\pm$ .002 (n = 4)	.0019 $\pm$ .004	.0051 $\pm$ .005 (n = 4)	.0053 $\pm$ .004	.0065 $\pm$ .004 (n = 4)	.0087 $\pm$ .004

(a) Significantly different between trypsin and control p = < .002 Mann-Whitney U Test.

to that which in the sheep was associated with an increased flux of lymphocytes - Smith, McIntosh & Morris (1970) revealed a pattern of localization of normal ^{51}Cr TDL that was similar to the DNCB[1] lesion although the early peak at $\frac{1}{2}$ hr was somewhat more obviously increased compared to normal skin and the element of late accumulation by 24 hrs was also consistently greater. The nadir at 2 hrs was however also a consistent feature. The localization of trypsin-treated ^{51}Cr TDL was again significantly impaired in this lesion at $\frac{1}{2}$ and 2 hrs (Table 5.12) with considerable recovery seen by 24 hrs. An absolute difference was still measured between localization of activity associated with control and trypsin-treated cells at this time but a parallel disparity in blood leucocyte ^{51}Cr activity in these particular recipients may explain this result. It was also noted that some degree of inhibition of localization of trypsin-treated lymphocytes was measured in gut, thymus and bone marrow.

In a preliminary experiment the mean concentration of ^{51}Cr in the subcutaneous mammary carcinoma nodule was .077% inj. act./gm at 24 hrs after iv injection of ^{51}Cr whole TDL which was considerably above that in normal skin. It was therefore decided to study the kinetics of localization of passaged ^{51}Cr tdl and ^{125}I Udr labelled lymphoblasts in this model system. The main reasons were that tumour nodules could be removed at biopsy allowing more than one time to be studied in each recipient, which is advantageous when the number of labelled cells that can be made available for injection is limited, as is the case with both these populations. Also the tumour is an abnormal tissue and therefore there is no true normal control with which comparisons can be made; thus no loss accrues to the failure to sample other tissues at the time of biopsy. Table 5.13 summarizes the results of two experiments in each of which duplicate recipients received a mixture of ^{51}Cr passaged TDL and ^{125}I Udr in vitro labelled TDL, and after $\frac{1}{2}$ + 2 hrs subcutaneous tumours were excised. At 24 hrs after cell injection the animals were killed with whole

TABLE 5:12

EFFECT OF TRYPSIN ON ^{51}CR LABELLED LYMPHOCYTE LOCALIZATION IN ADJUVANT GRANULOMA

	% INJECTED AVTIVITY/g ± S E					
	½ Hr n = 2		2 Hr n = 2		24 Hr n = 2	
	C	T	C	T	C	T
Normal skin	± .027 .006	± .037 .001	± .021 .005	± .025 .002	± .020 .002	± .016 .002
Granuloma n = 8	± .069(1) .004	± .038(2) .002	± .039(3) .002	± .021(4) .001	± .27 .02	± .10 .01
Blood leucocytes	± .42 .19	± .69 .08	± .10 .02	± .12 .003	± .21 .015	± .10 .034

Other tissues similar to Table 5.10

(1) vs (2) $p < .002$ Mann-Whitney U Test
(3) vs (4) $p < .002$ " " "

TABLE 5.13 THE KINETICS OF LOCALIZATION OF ^{51}Cr AND ^{125}I IN RECIPIENTS OF MIXTURES OF ^{51}Cr PASSAGED TDLs AND $^{125}Iudr$ IN VITRO TDLs

TUMOUR AND BLOOD SAMPLES AT BIOPSY AT ½ and 2HRS AND AFTER WHOLE BODY PERFUSION AT 24 HRS

Mean % of inj act./gm ± S.E. n = 4

TISSUE	½HR		2HR		24HR	
	$P^{51}Cr$	^{125}I	$P^{51}Cr$	^{125}I	$P^{51}Cr$	^{125}I
TUMOUR	.228 ± .08	.145 ± .014	.040 ± .004	.232 ± .05	.024 ± .005	.251 ± .04
BLOOD LEUCOCYTES	1.51 ± .2	.16 ± .04	.47 ± .08	.09 ± .01	.15 ± .01	.02 ± .001
SKIN					.001 ± .001	.06 ± .02

body perfusion as normal. With the passaged ^{51}Cr TDL population, peak values were measured at $\frac{1}{2}$ hr and at this time the blood level was still high. Levels in both these compartments fell with time but the fall in tissue was less marked than in the blood. With the ^{125}I-labelled TD lymphoblasts the fall in the blood level was much quicker and more profound. Concentration of ^{125}I in the tumour showed a slight increase with time. This pattern of localization of both isotopes was similar to that seen in both DNCB[1] and croton oil lesions with these populations.

The results of an experiment comparing both these populations with similarly labelled but trypsin-treated TDL (Table 5.14) suggested that there might be a slight degree of inhibition of localization of trypsinized lymphocytes in the tumour at $\frac{1}{2}$ hr, particularly if the blood concentrations were taken into account. This was most noticeable for the passaged ^{51}Cr TDL population. In order to assess if this inhibition was significant another experiment involved duplicate recipients of control or trypsin-treated ^{51}Cr passaged TDL each bearing several tumour nodules all of which were assayed after whole body perfusion at $\frac{1}{2}$ hr after iv injection of cells. In this experiment (Table 5.15) the blood levels of control and trypsin treated cells were similar and if one spuriously high control tumour value was discounted no difference was seen between the localization of either population in the tumour. Lymph nodes of these animals did show significant inhibition of localization of isotope associated with trypsin-treated passaged ^{51}Cr labelled lymphocytes, but little reduction in lymph node levels of ^{125}I was apparent.

Experiments of this type should be more easily carried out with ^{111}In-oxine labelled TDL as the higher cell specific activities obtainable would allow significant count rates with many fewer labelled cells and therefore more recipients could be studied with a given cell population.

Examinations of autoradiographs of tissue sections of

TABLE 5.14

THE LOCALIZATION OF ^{51}Cr AND ^{125}I IN TISSUES BIOPSIED AT $\frac{1}{2}$ AND 2HRS AFTER I.V. INJECTION OF ^{51}Cr PASSAGED TDL AND ^{125}I IN VITRO TDLs - EFFECT OF TRYPSINIZATION

Mean % inj./gm $\pm$ S.E. n = 2

TISSUE	$\frac{1}{2}$HR				2HR			
	$p^{51}Cr$		^{125}I		$p^{51}Cr$		^{125}I	
	CONT	TRYPS	CONT	TRYPS	CONT	TRYPS	CONT	TRYPS
TUMOUR	.296 $\pm$ .08	.172 $\pm$ .02	.156 $\pm$ .02	.148 $\pm$ .01	.045 $\pm$ .01	.048 $\pm$ .01	.201 $\pm$ .04	.195 $\pm$ .002
BRACHIAL LYMPH NODE					13.2	1.8 $\pm$ .19	.41	.62 $\pm$ .1
BLOOD LEUCOCYTES	1.4 $\pm$ .02	1.83 $\pm$ .28	.15 $\pm$ .28	.29 $\pm$ .05	.58 $\pm$ .11	.44 $\pm$ .02	.44 $\pm$ .05	.10 $\pm$ .02

TABLE 5.15

Localization of ^{51}Cr in Tissue $\frac{1}{2}$ hr after iv Injection of ^{51}Cr-passaged TDLs (Perfused)

% Inj.act/gm ± SE

Tissue	Control	Trypsin
Tumour n=7	.368 ± .185 excluding one high value - (∴ n = 6) .183 ± .027)	.199 ± .03
Cervical Lymph Node	4.7 ± 1.0	.25 ± .02
Blood Leucocytes	4.2 ± .4	3.8 ± .4

inflammatory lesions following skin painting, or injection of adjuvant, and of the tumour, showed occasional labelled cells after injection of either tritiated uridine or tritiated thymidine in vitro labelled TDL but the frequency was too low to allow meaningful quantitation.

DISCUSSION

Examination of the kinetic localization data in a way similar to that for normal tissues suggests that there is a net increase in the proportion of the total lymphocyte pool at equilibrium in specific recall contact sensitivity lesions, and areas of inflammation following application of croton oil, or injection of Freund's adjuvant. The shape of the distribution curve for ^{51}Cr whole TDL suggests that in inflammatory sites the peak is still very soon after injection with usually some degree of trough at 2 hrs and only relatively small further increases by 24 hrs. The observation, that the bulk of any increased localization occurs so early, is consistent with the idea that accumulation of lymphocytes follows an increased rate of extravasation rather than a decreased rate of exit. The slight trough at the time of lowest blood concentration suggests that at least a proportion of the extravasated cells have already left the tissue by this time and therefore have a rapid transit time. However the slight degree of late accumulation in specific recall DNCB lesions may reflect some degree of increase in the modal transit time through the tissue in a way analogous to lymph nodes. A similar pattern holds for the croton oil irritant lesion while for the 'primary' lesion following painting with sensitizing agents little change from normal skin can be discerned.

In the granuloma following injection of Freund's complete adjuvant the degree of late accumulation is even greater than for the contact sensitivity lesion and can be interpreted as suggesting both an increased 'clearance factor' of lymphocytes across the endothelium and a prolongation

of the modal transit time.

These results for contact sensitivity lesions are similar to those of Sabbadini et al (1974). Their results also showed that the bulk of any increased localization of ^{51}Cr lymph node cells in mouse ears specifically challenged with Picryl chloride was present by 3 hrs after iv injection provided that the lesion was 24 hrs old. On the other hand no increased localization over normal skin was present 3 hrs after injection if the challenge was given at the same time as the cells, but a normal degree of localization was present by 24 hrs under these conditions. This latter observation was confirmed in the experiment shown in Table 5.5 in which normal degrees of increased accumulation were present at 24 hrs and later even though the challenge was not given until three hours after the labelled cells were injected. This argues strongly against the idea that the early extravasation of cells following injection of a 'bolus' of cells with consequent artificially high blood levels for a short period could be the cause of the late accumulation. In the experiment described the blood level of labelled cells at the time of skin challenge would be minimal and therefore such a mechanism of early localization could not operate. It is very likely therefore that there is a flux of lymphocytes through inflammatory lesions of increased magnitude but of a similar rapid time course to that in normal non-lymphoid tissues.

As in the case of normal tissues, the question arises of whether this increased flux is due to the minority population of lymphoblasts contained in normal TDL. Several pieces of evidence combine to suggest that this subpopulation is not entirely responsible for the activity measured even though many workers have reported that 'activated' lymphocytes have a particular propensity to accumulate in inflammatory exudates and infiltrates (Werdelin 1972, Allwood 1975, Rose et al 1976, Moore & Hall 1973, Koster et al 1971). Firstly, the difference

in shape of the kinetic distribution curves (data summarized in Fig 5.3) obtained with ^{125}I Udr-lymphoblasts and ^{51}Cr whole TDLs, particularly between $\frac{1}{2}$ and 2 hrs in skin painted with irrelevant antigens suggests that all of the ^{51}Cr activity cannot be accounted for by the cell type that is also labelled with ^{125}I Udr. The observation of some increased localization of ^{51}Cr associated with passaged ^{51}Cr TDL at 24 hrs suggests that some accredited recirculating lymphocytes can enter recall contact sensitivity lesions and those induced by croton oil. Localization of long-lived recirculating cells in a cell-mediated immune lesion is also suggested by the increased concentration of tritium associated with long-lived ^{3}H-Tdr in vivo labelled TDL in DNCB[1] lesions but with this label no increase was measured in the non-immune irritant lesion with croton oil. Also the reduction of localization of trypsin-treated ^{51}Cr labelled cells in skin lesions and lymph nodes is not paralleled by the behaviour of ^{125}I-Udr labelled lymphoblasts in either situation.

The similarity in the ratio of % inj.^{125}I/% inj.^{51}Cr in recipients of both populations in the DNCB[1] lesion and normal skin also suggests that the increased flux through the cell-mediated immune lesion includes all cell types and if the considerations taken into account in the last chapter are true then approximately equal numbers of small recirculating lymphocytes and lymphoblasts may comprise the infiltrate.

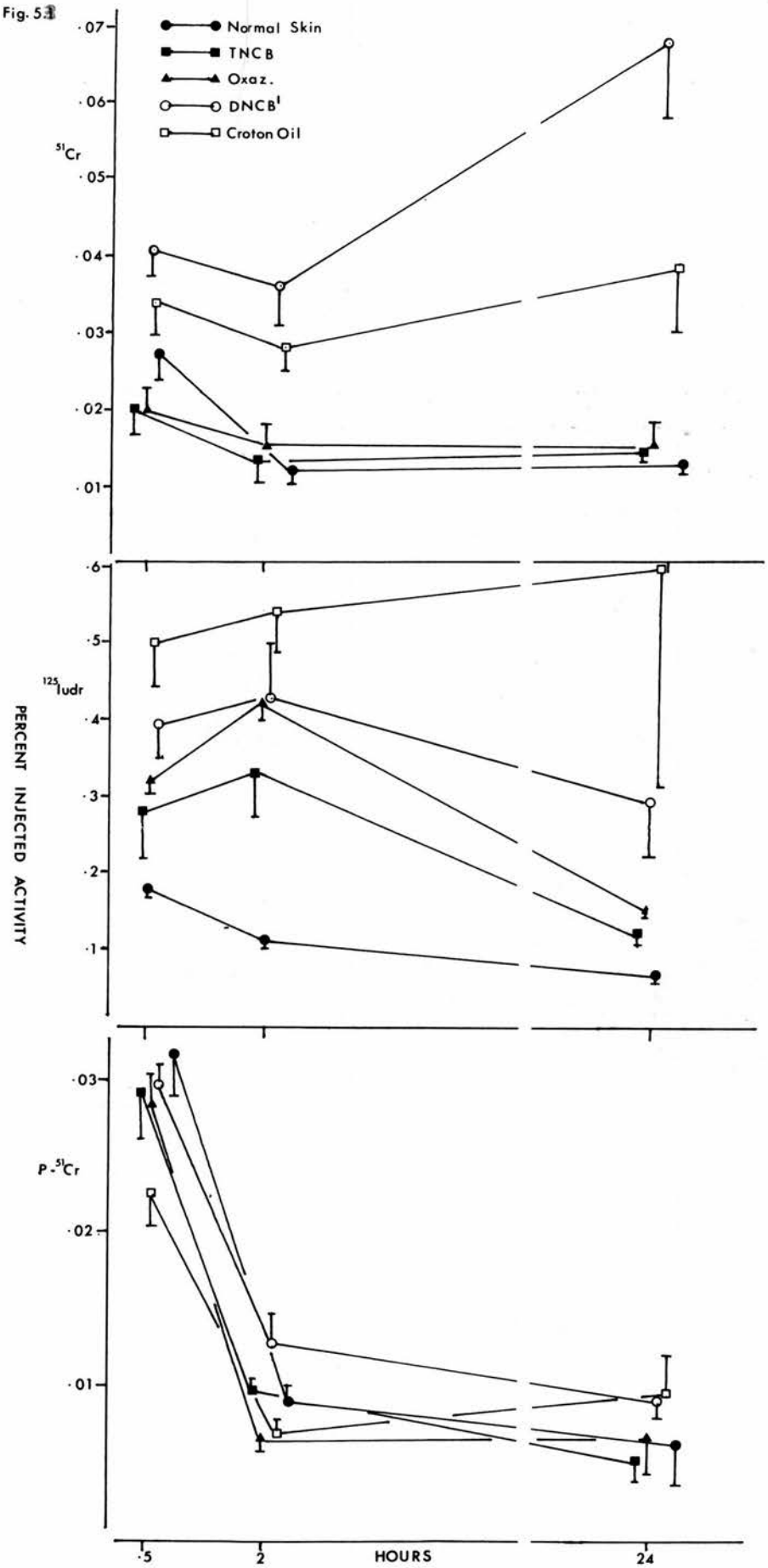
The general impression that emerges is, therefore, that in simple irritant lesions following painting with croton oil or contact sensitizing agents in non-sensitized animals there may be a small increase in lymphocyte flux with a higher proportion of lymphoblasts than in normal tissues, but in fully developed cell-mediated lesions small long-lived lymphocytes are also present and the total flux is increased several-fold.

The inhibition of localization of trypsin-treated lympho-

FIG 5.3

The distribution of radioactivity in normal and inflamed skin at intervals after iv injection of syngeneic labelled TDL. Summary of data given in Tables 5.4, 5.6, 5.7.

Fig. 53


Normal Skin
TNCB
Oxaz.
DNCB¹
Croton Oil
^{51}Cr
$^{125}Iudr$
P-^{51}Cr
PERCENT INJECTED ACTIVITY
·07
·06
·05
·04
·03
·02
·01
·6
·5
·4
·3
·2
·1
·03
·02
·01
·5
2
HOURS
24

cytes in contact sensitivity and granuloma lesions is almost total if it is acceptable to take normal skin concentrations as relevant background values. This result strongly suggests that the increased extravasation in cell mediated immune lesions follows some qualitative change in the endothelium that increases the efficiency of extravasation of lymphocytes and possibly makes the endothelium appear more like the specialized post-capillary venular endothelium of lymph nodes to the lymphocytes in the blood. It is therefore unlikely that the increase in extravasation results solely from hyperaemia even though the extent of this change in blood flow has been reported to be great enough to account for the increase in lymphocyte influx into the tissues without any increased efficiency of the extravasation mechanism (Hay et al 1977).

Until the precise mechanisms of interaction between lymphocyte and venule endothelium in post-capillary venule and in sites of cell-mediated inflammation are discovered, it is impossible to say that they are identical but they do both appear to be trypsin sensitive. In the 24 hr old recall contact sensitivity lesion no morphological evidence of venular endothelium specialization was seen although cuboidal change in this type of vascular endothelium has been reported (i) in parts of longstanding subcutaneous granulomata following injection of virus + Freund's complete adjuvant in the sheep (Smith et al 1970), (ii) in the thymus of some myasthenia gravis patients (Bradfield 1973), (iii) in areas of dense small lymphocyte infiltration of muscles in some patients with polymyositis (Ball J, pers.comm.) and associated with lymphocyte infiltration of a human medullary carcinoma of breast (Ahmed A. pers.comm.), (iv) in an experimental arthritis in rabbits injected repeatedly with horseradish peroxidase (Graham & Shannon 1972), (v) in experimental chronic inflammatory lesions in the chicken and rheumatoid arthritis lesions in humans (Miller J J 1969).

These observations suggest that some change does occur in

the function of vascular endothelial cells in areas of inflammation and that there could possibly be a morphological change that occurs if the stimulus is maintained for long enough. An extension of this idea suggests that the specialization of the post-capillary venules in lymph nodes followed some change that rendered this 'inflammatory' modification non-reversible in certain areas around which organized lymph node structures evolved. Support for this theory comes from the fact that organized lymph nodes developed later in phylogeny than lymphocytes, lymphocyte recirculation, or cell-mediated immune inflammatory reactions, and are in fact only present in mammals.

This idea that chronic inflammatory areas behave as lymph node analogues is supported by the results pertaining to the adjuvant granuloma which showed an increase in flux and also some lengthening of the interstitial transit time. The afferent lymph output in the sheep draining such a lesion had the magnitude and differential cell count that would normally be expected for the efferent lymph draining a lymph node of the same weight as the granuloma (Smith et al 1970).

The situation as far as the subcutaneous nodule of mammary carcinoma is concerned is less clear. The limited series of experiments suggests that such tissue had a greater flux than normal skin but this may not be a relevant comparison. The equivocal results obtained with trypsin-treated lymphocyte localization suggest that this flux does not depend upon a trypsin sensitive mechanism and may therefore suggest that there is not a cell-mediated immune inflammatory component to the lesion. This may indeed be the case as the tumours grow freely in syngeneic and F_1 hybrid female recipients with no evidence of rejection.

The questions that still remain unanswered are: Is peripheral sensitization an important mechanism of initiating immune responses? Which cell types are the most important for triggering immune inflammation and where is the

precise location of interaction with antigen? The relatively greater ability of recently formed lymphocytes to extravasate in all tissues could perhaps provide a great enough frequency of antigen reactive cells if these were confined to this minority population in the blood. In primary immune responses 'virgin' B lymphocytes may belong to this subpopulation (Strober 1969, 1972) and if the irritant action of any antigen administration preferentially allows increased entry of this cell type into the tissues then peripheral sensitization might be expected (de Sousa & Parrott 1969, Macher & Chase 1969). This type of effect will also explain the direct development of delayed type hypersensitivity reactions (eg 4-5 days after sensitization with TNCB) in the mouse in the site of primary application of antigen that is almost certainly mediated by activated T lymphoblasts generated in the draining lymph node (Allwood 1975).

However, as mentioned already, the triggering of this type of cell-mediated response in long-term immunized subjects can only be explained by interaction of antigen with long-lived memory cells that will be of the classical small recirculating type. A precise knowledge of the frequency of these various cell types that can react to a given antigen would help to clarify whether fluxes of the order suggested are great enough to account for the development of the reactions.

CONCLUSIONS

Lymphocyte fluxes through skin were increased by eliciting cell-mediated immune responses and this flux probably comprised the same mixture of cell types as in normal non-lymphoid tissues.

The increase in flux through irritant lesions may have an increased proportion of lymphoblasts.

In cell-mediated immune inflammation the increased entry of lymphocytes is trypsin-sensitive and from this point of

view is similar to the very efficient entry of lymphocytes into lymph nodes. The entry into irritant lesions is possibly not trypsin-sensitive.

The tumour model studied probably showed no evidence of a cell-mediated immune reaction.

CHAPTER SIX

The Bone Marrow - A Secondary Lymphoid Organ

Introduction

The bone marrow is the major site of haemopoiesis in the adult mammal. The generation in this tissue of erythrocytes, polymorphonuclear leucocytes, monocytes and platelets (from megakaryocytes) are all well established phenomena. In addition, bone marrow populations contain small round cells with the morphological appearance of lymphocytes. Following studies using systemic and local administration of tritiated thymidine it is clear that many of these bone marrow lymphocytes arise by cell division at this site (Everett et al 1964, Brahim & Osmond 1970). With the development of surface markers for sub-populations of mature lymphocytes it was shown that only a small minority of bone marrow cells contained surface θ antigen in mice. That such mature T cells though needing the thymus for inductive maturation were ultimately derived from bone marrow precursor cells was shown by re-population studies after lethal irradiation, in which this latter source was essential for the full recovery of T cell populations (Claman 1974). Also fully mature B cells with dense surface immunoglobulin (Ig) comprised only a small proportion of bone marrow lymphocytes (Osmond & Nossal 1974a). Using timed administration of ^{3}H Tdr and also scoring cells for surface Ig (Osmond & Nossal 1974b) showed that there was indeed a maturation of newly formed bone marrow lymphocytes with accumulation of surface Ig in increasing density. The time of release of such developing B cells into the blood appeared random with respect to an individual cell's maturity as assessed by surface markers.

Because of these results of a high labelling index of marrow lymphocytes with ^{3}H Tdr after only 3 days administration, showing that most cells are newly formed, and the low proportion of cells having surface markers typical of mature long-lived lymphocytes, it was considered that there was only a minor interchange between the blood and marrow of recirculating lymphocytes. This idea was reinforced by the relatively low immunocompetence of bone marrow cell populations. However, the presence of some functional activity known to require T cells or memory B cells in this compartment and the ability to reduce this functional activity by prolonged thoracic duct drainage argued in favour of some recirculation through this tissue. Such functional markers were (i) the graft-versus-host (GVH) activity of rat marrow cells (Yoshida & Osmond 1971) that was shown to be carried by small lymphocytes by cell size fractionation and is established to be almost exclusively the property of small T cells in the rat popliteal lymph node assay used (Ford et al 1970, Rolstad & Ford 1974). Similarly the GVH activity of mouse marrow cells was abolished by treatment with anti-θ serum (Cantor 1972); (ii) the adoptive primary antisheep erythrocyte haemolysin response of bone marrow cells (Howard & Scott 1972)measured in irradiated recipients challenged with antigen was much reduced in donors subjected to prolonged thoracic duct drainage; (iii) mature T cells were notably absent in the bone marrow of nude mice and were markedly diminished in neonatally thymectomized mice (Claman 1974).

Indirect evidence supporting this blood-marrow interchange of immuno-competent lymphocytes comes from the observation of increased functional activity of marrow populations with increased proportions of mature T cells as shown by surface markers or ^{51}Cr labelling in mice treated with hydrocortisone. These same animals showed a deficit of immuno-competence in the blood and lymph nodes at the same time (Moorhead & Claman 1972). Similar results obtained in the guinea pig (Fauci 1975).

The observations of Gordon et al incidentally provided more direct evidence of both release from and uptake into the bone marrow of lymphocytes in an isolated rat femur preparation. The experiments were designed to measure the kinetics of polymorph release under the influence of releasing factors but the differential counts of the input and output perfusates showed (i) a deficit of lymphocytes in the output if lymphocyte rich blood was used as perfusing fluid, but (ii) if cell free plasma was used then lymphocytes were found in the outflow perfusate and these must have derived from the marrow. Histological examination showed no disruption of the marrow architecture that would account for artefactual release of cells under the conditions used (Gordon et al 1964).

Histological examination with the electron microscope also revealed lymphocytes in transit between blood and marrow parenchyma between sinusoidal endothelial cells, but the direction of movement could not be deduced from these static preparations (Hudson & Yoffey 1966).

The data presented in Chapter 4 suggested that there is a substantial flux of lymphocytes between blood and bone marrow. Labelling with ^{51}Cr or ^{125}I Udr supported the thesis that both large and small lymphocytes could use this pathway with a modal transit time similar to or shorter than that in the spleen. The size of this flux had not been appreciated previously because of the diffuse nature of the organ and also because early time intervals when peak values are found were not studied.

The experiments to be detailed in this chapter were designed to investigate further the relationship between immuno-competent lymphocytes and the bone marrow. Functional markers were used to follow the migration of thoracic duct lymphocytes thus avoiding the potential toxicity associated with radioactive labelling. The capability of the marrow organ to act as a lymphoid tissue was assessed by measuring the localization of labelled

particulate antigen, and by investigating the behaviour of injected parental strain lymphocytes in F_1 hybrid recipients in which a systemic graft versus host reaction would develop, in order to determine whether this site could produce specific antigenic retention of reactive lymphocytes with blast-transformation and proliferation as is known to occur in the spleen (Gowans 1962, Ford et al 1975).

Methods

1 Bone Marrow Cell Preparation

Both femora, tibiae and humeri were removed immediately after death and placed in PBS containing a final concentration of 5 mM EDTA and 15% FCS on ice. The marrow was flushed from the marrow cavity, after perforation of the epiphyseal plate using a 19 or 21g needle, with approximately 10ml of the EDTA/PBS/FCS per bone, as soon as possible. The fragemnts of marrow parenchyma were broken by repeated aspiration with a pasteur pipette followed by sieving through a sterile stainless steel wire mesh. The resulting suspension was allowed to stand in 35ml conical tubes held vertically on i ce for at least 10 mins. Large particles sedimented quickly and the supernatant single cell suspension was aspirated with a pasteur pipette, taking care to avoid the inevitable surface debris. The cells were then washed once by centrifuging at 350g for 10 mins at $4^{o}C$ and resuspended for counting and injection at suitable concentrations in PBS. Troublesome clumping of cell suspensions after washing was avoided by maintaining the cells on ice at all times. EDTA was used as an anticoagulant, as bone marrow is rich in tissue thromboplastins and also in the hope that it might encourage dissociation of reactive cells from their antigenic tissue in a way analogous to the detachment of cytotoxic effector cells from specific target cell monolayers to which they adhere (Stulting & Berke 1973).

2 Irradiation

Whole body irradiation was used to greatly reduce the immune competence of final recipients in the adoptive memory antibody response assay for memory B & T cells. The dose usually of 750 rads was administered to rats restrained in 4.4cm wide space within perspex cages with 12 Mev electrons from a horizontally directed linear accelerator (pulse width 50 μ sec repetition rate 50 Hz current 3 amps peak). Recipients in the adoptive antibody assay received transferred cells on the day of irradiation.

Radiation of intermediate recipients with up to 1000 rads was used in attempts to reduce the background GVH reactivity of bone marrow.

3 Assay for the adoptive transfer of memory to Human Serum Albumin (HSA).

The transfer of lymphocytes from rats, primed by the injection of alum-precipitated HSA together with 8×10^9 Bordetella pertussis organisms at least 3 months previously, to irradiated syngeneic recipients which were then challenged with 10 μg soluble HSA 24 hrs later, allowed the production of a secondary antibody response whose magnitude was directly related to the number of lymphocytes transferred (Bell & Shand 1975). The antigen binding capacity of serum taken on days 14 and 21 after challenge was measured using a modified Farr-type radio-immuno-assay.

Serum samples were assayed for antigen binding capacity (ABC μg antigen bound/ml of neat serum) and relative affinity ('S') by the ammonium sulphate precipitation test using ^{125}I-HSA monomer as described by Bell & Shand (1975). Dilutions of antisera, in duplicate, were made with a Micromedic automatic pipette (Micromedic System Inc, Phila, Pa) and ABC determined both at 0.1 μg and 10 μg HSA monomer/ml. However, when the ABC was 2.0,

the sample was also tested at 1.0 μg HSA/ml. A computer programme was written to calculate the relative affinity and when necessary to extrapolate the ABC at 1 μg/ml.

4 ^{51}Cr Labelling of Sheep Erythrocytes

Sheep erythrocytes (Tissue Culture Services, Slough) were washed twice in PBS and resuspended at a concentration of 5 x 10^8/ml in RPMI + 10% FCS and incubated with Na_2 ^{51}Cr O_4 at 50 μCi/ml at 37°C for 1 hr. The labelled cells were washed three times in 40ml vols of PBS and resuspended for injection. The dose of cells administered iv was adjusted to give adequate total activity.

5 Popliteal Lymph Node Assay of Graft versus Host Activity

The method of Ford Burr & Simonsen (1970) was used, in which graded doses of cells were injected subcutaneously into the footpads of young (less than 8 weeks old) F_1 hybrid rats. Each population was assayed in quadruplicate at two doses. For bone marrow suspensions where large cell doses were required an injection volume of 0.2ml was usually employed and if this was the case all populations within the experiment were injected in this volume rather than the standard 0.1ml volume. Seven days later the draining popliteal lymph nodes were removed, dissected clear of adherent fat and weighed to ± 0.1mg.

Results

A Localization of Particulate Antigens

Tables 6.1 and 6.2 show the results of kinetic distribution studies of ^{51}Cr-labelled xenogeneic erythrocytes and heat-killed syngeneic thoracic duct lymphocytes respectively. Comparison of the localization of radioactivity expressed as the percentage injected dose in animals perfused at 1 or 2 and 24 hrs after iv injection of labelled cells showed that the

TABLE 6.1

The Distribution of Radioactivity in Recipients 1 & 21 Hrs after i v injection of 8×10^8 ^{51}Cr-labelled sheep erythrocytes.

(Triplicate recipients 'perfused' at time of sampling)

Mean % injected activity per whole organ ± S E.

Tissue	1 Hr	21 Hrs
Spleen	1.3 ± .03	2.7 ± .1
Bone marrow	3.5 ± .3	4.2 ± .3
Liver	33.3 ± 1.5	41.6 ± .7
Lymph node (superficial)	.17 ± .02	.13 ± .02
Skin	3.7 ± .53	1.8 ± .08
Small intestine	1.1 ± .11	.62 ± .08
Lung	1.1 ± .21	.4 ± .002
Blood leucocytes	1.7 ± .4	.13 ± .01
Plasma	26.5 ± .7	2.2 ± .3

TABLE 6.2

Tissue Distribution of Radioactivity 2 Hrs & 24 Hrs after i v Injection of 130 x 10^6 ^{51}Cr-labelled Heat-Killed Syngeneic TDL

Mean % injected activity per whole organ ± S E (n = 2)

Tissue	2 Hr	24 Hr
Spleen	1.9 ± .17	2.7 ± .18
Bone marrow	6.1 ± .16	11.1 ± .9
Liver	57.1 ± 1.0	58.6 ± 2.6
Lymph node (superficial)	.06 ± .002	.11 ± .02
Skin	3.0 ± .2	2.2 ± .02
Small intestine	.81 ± .06	.42 ± .06
Lung	34.9 ± 12.5	2.4 ± .16
Blood leucocytes	.62 ± .3	.36 ± .07
Plasma	7.6 ± .06	1.6 ± .17

majority of the injected activity was in the liver at both times. The high early level in the lung had fallen by 24 hrs and presumably reflects continued clearance of cells from the blood. The cell-free label in the plasma showed a similar pattern, as did the activity in most non-lymphoid tissues as exemplified by skin values. Lymph nodes accumulated very little activity at any time. In contrast to these tissues the spleen and bone marrow showed significant localization at two hours but also showed increased activity by 24 hrs. These results are consistent with an interpretation that the activity in these sites is due at least in part to the phagocytic activity of the rich population of macrophages lining the blood sinusoids and therefore supports the idea that the marrow could be a site of antigen concentration.

B Migration of TDL to Syngeneic Bone Marrow

Fig 6.1 and Table 6.3 summarize the results of experiments in which the migration of syngeneic cells into and out of the marrow was assessed with functional markers.

The protocol for these and several subsequent experiments was to inject groups of intermediate recipient rats intravenously with thoracic duct lymphocytes either two or twelve hours before making single cell suspensions of the bone marrow of these and uninjected controls. These marrow populations and the original TDL were then used in functional assays using appropriate final recipients.

Using GVH reactivity of parental strain lymphocytes in F_1 hybrid recipients as a measure of T cell function, triplicate groups of parental HO B2 strain rats were injected with 350×10^6 syngeneic TDL. The marrow populations were taken after intervals of zero, 2 and 12 hrs and assayed in HO B2 x PVG/c F_1 hybrids using the popliteal lymph node assay. It is clear (Fig 6.1)

FIG 6.1

Ex 103. 350×10^6 $HO.B_2$ TDL were transferred to each member of groups of three intermediate normal rats by iv injection either 2 or 12 hrs before death. Bone marrow cell suspensions were prepared from the six major long bones of each recipient and the suspensions from each group of syngeneic or $HO.B_2$ x Pvg/C F_1 hybrid intermediates were pooled for each time period. These suspensions together with those made from matched but uninjected control groups of rats and a sample of the $HO.B_2$ TDL were then assayed for GVH activity in $HO.B_2$ x Pvg/C F_1 hybrids using the popliteal lymph node assay.

Fig 6.1 shows the dose response relationships of each population for the parental strain syngeneic groups of rats.

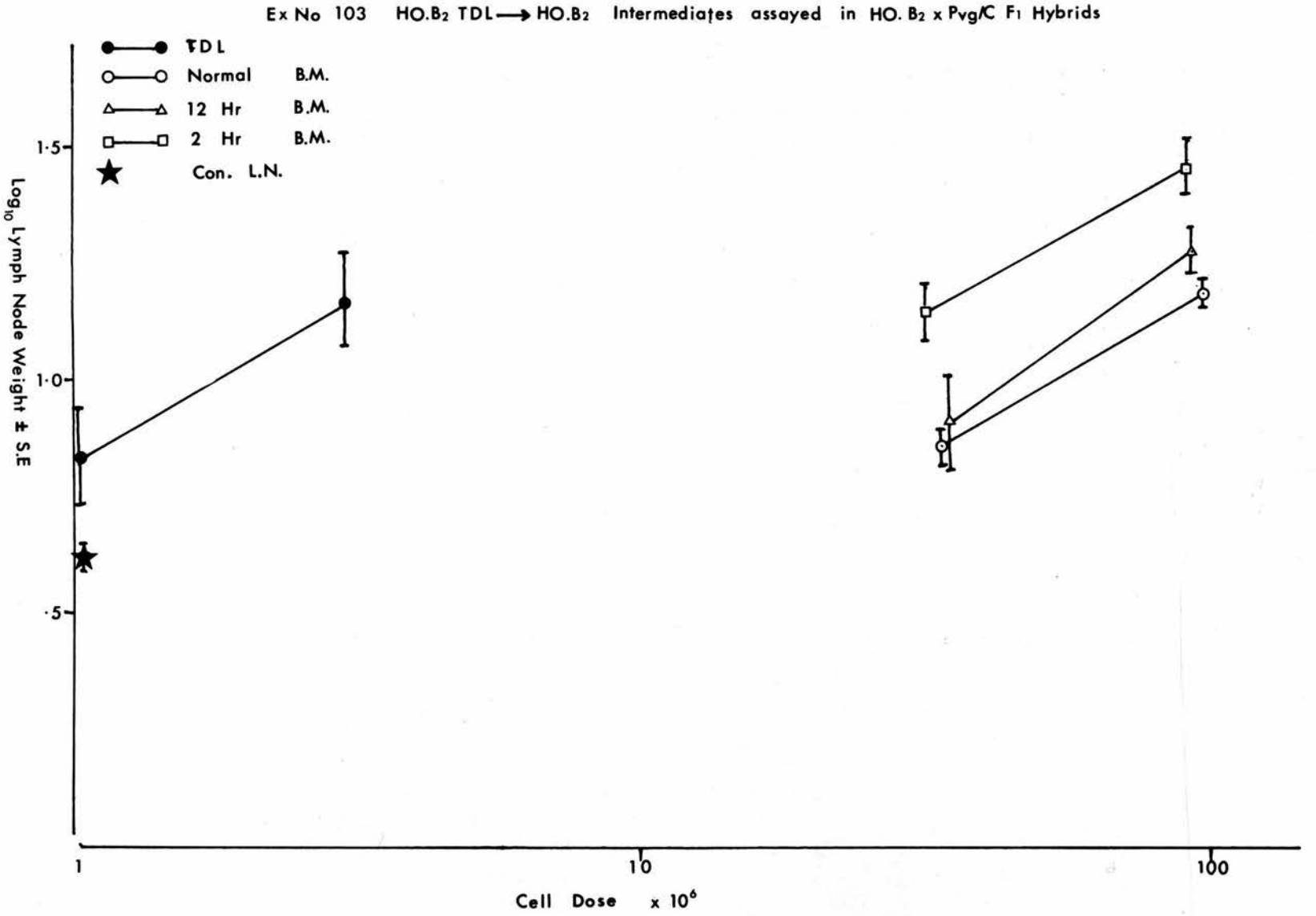

Ex No 103 HO.B2 TDL ⟶ HO.B2 Intermediates assayed in HO. B2 x Pvg/C F1 Hybrids
TDL
Normal B.M.
12 Hr B.M.
2 Hr B.M.
Con. L.N.
1·5
1·0
·5
Log10 Lymph Node Weight ± S.E
1
10
100
Cell Dose x 10^6

TABLE 6.3

Geometric Mean Antigen Binding Capacity ($\pm$ SE) of Sera of Groups of Five (750 RAD) Final Recipients 21 Days after I.V. Injection of Bone Marrow Cells derived from Duplicate Intermediate Normal or 800 RAD Irradiated Rats which had received Syngeneic TDL from HSA-Primed Donors, 2 or 12 Hrs Previously.

(Each final recipient received $^1/5$ of the total cells collected from 6 long bones of both intermediates).

2 Hrs		12 Hrs	
Normal	750 rad	Normal	750 rad
2.2 ($\pm$ .06)	4.6 ($\pm$ .15)	0.3 ($\pm$ .16)	0.8 ($\pm$ .16)

that the potency of bone marrow cells, though much lower than pure TDL, is significantly raised 2 hrs after a bolus injection of TDL compared to uninjected bone marrow, but has dropped almost to background levels by 12 hrs.

Also using TDL from HSA-primed donor PVG/c rats which include memory B and T cells and assaying the bone marrow of intermediate syngeneic recipients for the ability to produce an adoptive secondary antibody response to a standard dose of soluble antigen in irradiated final syngeneic recipients, a similar pattern occurred. The antigen binding capacity of day 21 sera of the group of rats receiving bone marrow from intermediates injected with TDL 2 hrs previously was much greater than that measured in final recipients of bone marrow cells from intermediates injected with TDL 12 hrs previously. Normal and irradiated intermediates were both used in case there was significant suppression of the adoptive response by normal bone marrow cells. The presence of suppressive activity in the marrow could explain the slightly higher response in irradiated animals, but another explanation is suggested by the results shown in Table 6.4, in which the distribution of ^{51}Cr-labelled syngeneic TDL was compared in similar pairs of irradiated and normal recipients. In the irradiated recipients the concentration of label associated with blood leucocytes dropped much more quickly than in intact controls and remained at lower levels even by 24 hrs. There was a considerable excess of activity in the bone marrow of irradiated recipients at 2 hrs and this coincided with a deficit in the spleen. This reciprocal imbalance had disappeared by 24 hrs. There was no difference between lymph node localization on a whole node basis, although an increase in concentration (counts/gm) was noted in the nodes 1 day after irradiation as the nodes were very shrunken by this time. The same shrinkage was

TABLE 6.4

Tissue Distribution of Radioactivity 2 & 24 hrs after iv Injection of 120 x 10^6 51^{Cr}-labelled TDLs into Duplicate Normal or 750 RAD Syngeneic Rats (perfused at time of sampling).

Percent Injected Dose per Organ (SE for most tissues was less than 10% of the mean and has been excluded for the sake of clarity).

Tissue	2Hrs		24Hrs	
	Normal	750 rad	Normal	750 rad
Spleen	37.0	22.5	17.5	16.6
Bone marrow	25.2	35.0	4.7	5.6
Lymph node (superficial cervical)	2.3	2.6	6.0	8.0
Intestine	19.5	23.8	15.2	18.4
Thymus	.03	.13	.07	.14
Lung	5.2	5.8	2.3	1.6
Liver	10.0	10.1	10.1	9.0
Skin	.3	.3	.31	.31
Muscle	.6	.55	.6	.6
Brain	.049	.013	.007	.005
Blood leucocytes	.69	.18	1.5	.17
Plasma	.38	.23	.40	.26

seen in the thymus but here the increased concentration was still noted on a whole organ basis and may reflect some degree of inflammatory change associated with the massive cell death in this tissue. The splenic activity was reduced overall but showed normal concentrations on a weight basis. The net redistribution of activity at 2 hrs from spleen to bone marrow could be the result of two factors. Firstly, the shrunken spleen may have a reduced total blood flow, which with a normal clearance factor would result in a net deficit - a partial splenectomy that would allow more cells to be available for extravasation elsewhere. One observation against this suggestion is the concurrent deficit in the blood that argues more in favour of competitive removal of labelled lymphocytes from the blood by other tissues of which the most important is obviously the bone marrow. That this might be likely is suggested by the histological appearance of bone marrow 24 hrs after irradiation; at this time there is marked loss of parenchyma and wide dilation of the blood sinuses. The latter may simply be due to compensatory dilation because of the tissue shrinkage within a rigid bony case, but would result in an increased blood supply of lymphocytes and therefore increased extravasation even in the absence of any increase in clearance factor. Thus on a per whole organ basis, which was used to assay the HSA-memory response, higher values would be expected at 2 hrs in irradiated compared to control bone marrow simply as a reflection of the greater number of extravasated lymphocytes.

The pattern of localization in syngeneic bone marrow was essentially similar when measured with functional markers as that seen with radiolabels. Precise quantitation in terms of the proportion of the injected dose localized in the tissues was impossible with these functional assays for several reasons. The injected TDL populations, although from the same panel of donors

was collected over different periods to allow staggered injections and therefore simultaneous preparation of bone marrow cell suspensions. The potency of the populations could therefore differ slightly and calibration against the TDL starting population has only limited accuracy. Perhaps more important was the likely variation between the localization in individual recipients and also the inconsistency of efficiency of removal of bone marrow for preparing single cell suspensions.

C Migration of TDL to Semiallogeneic Bone Marrow

The behaviour of injected parental strain TDL in F_1 hybrid recipients was studied in several ways. Ford et al (1975) and Atkins & Ford (1975) showed retention of specifically reactive parental lymphocytes in the spleens of F_1 hybrids by (i) using alternative radio-labelling techniques which allowed comparison of the distribution of responsive and non-responsive populations in the same recipient and (ii) showing a retention of ^{3}H-Udr labelled parental TDL with blast transformation in histological autoradiographs of the spleen. This antigen-reactive cell retention occurred within 24 hrs and was associated with complete removal of the relevant GVH activity from the recirculating lymphocyte pool as sampled in the thoracic duct. In the spleen the GVH reactivity of single cell suspensions made 24 hrs after injection of parental TDL did not show the expected increase in potency of GVH reactivity (Atkins & Ford 1975). It was suggested that this may have been at least partly attributable to the failure of transformed cells to enter the suspension.

The size of retention in the spleen and the relatively less marked retention seen in lymph nodes was entirely consistent with the idea that the reactive lymphocytes remained in the tissues in which they first extravasated. It was therefore considered likely that a

similar phenomenon would take place in the bone marrow if this tissue could be recognized as antigenic and could support immunological blast transformation and cell division.

Table 6.5 shows the result of an experiment in which parental A0 TDL were labelled with ^{3}H-Udr in vitro and injected into irradiated syngeneic or A0XDA F_1 hybrid recipients which were killed 2 or 24 hrs later. The recipients were perfused with PBS and then 1% Glutaraldehyde as a tissue fixative. The cleaned fixed long bones were then decalcified and prepared for routine histological sectioning.

Autoradiographs were then prepared and the total number of labelled cells and the proportion of pyroninophilic blast cells per unit area of cellular marrow parenchyma were assessed. The recipients were irradiated to reduce the resident proliferative population to facilitate scoring of labelled cells. This procedure incidentally may also have accentuated the early localization as suggested by Table 6.4.

At 2 hrs after injection similar numbers of labelled cells were found in both strains of recipient but by 24 hrs there was not only significant retention of labelled cells but also a marked increase in the proportion of blast cells in the F_1 hybrids. Occasional mitoses of labelled cells were also noted in the F_1 hybrids. Thus by 24 hrs there was a seven-fold increase in blast transformation in the F_1 hybrid confirming the ability of parental strain TDL to instigate a systemic GVH reaction in the bone marrow and also showing that the bone marrow can provide a suitable environment for such cellular activity.

The next approach was to use the same experimental design as that described for syngeneic animals to assay the GVH reactivity of F_1 hybrid bone marrow populations after iv transfer of parental strain TDL in an attempt to measure any functional retention of specifically

TABLE 6.5

The Distribution of ^{3}H-Udr Labelled AO TDL in AO or AO x DA F_1 Hybrid Bone Marrow 2 Hrs & 24 Hrs after iv Injection

(Frequencies derived from counts of approx. 1000 labelled cells at 2 hrs and 200 labelled cells at 24 hrs in sections of six bones from duplicate recipients of each type at each time period).

Strain	2 Hrs		24 Hrs	
	Labelled Cells/Unit Area	% Blasts	Labelled Cells/Unit Area	% Blasts
AO (syngeneic)	39	2.4	2.4	10
AO x DA (F_1 hybrid)	47	2.7	6.2	24

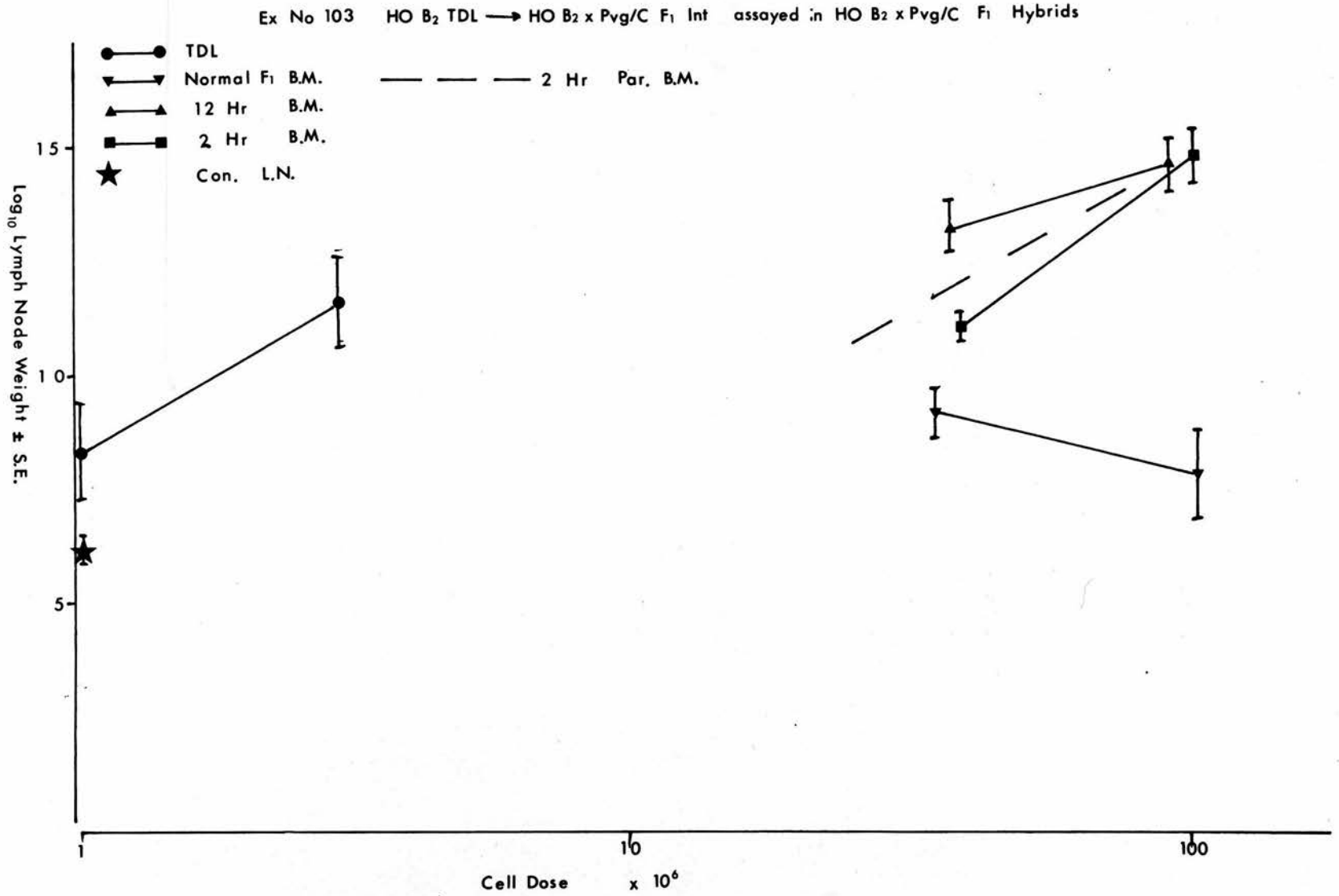
reactive cells. The demonstration of such positive selection has proved more difficult than showing the absence of a reactive population. Thus as mentioned no increase in potency of GVH reactivity was found in F_1 hybrid spleens. Similar difficulties were also encountered when specific retention of sheep erythrocyte reactive cells were looked for in the spleens of mice soon after antigen injection (Sprent & Miller 1973). Explanations for these failures included the possibilities that transforming cells were not well represented in single cell suspensions or that even if they were they either did not migrate normally or were refractory to further antigenic challenge in the final assay recipients. In attempts to obviate these factors 12 hrs was chosen as a 'late' time because morphological blast transformation is not apparent as early as this but most of the cells initially present in marrow have emigrated again by this time. Also EDTA was included in the medium used to isolate bone marrow cells both as an anticoagulant and as an agent that is known to cause the detachment of cytotoxic effector cells from monolayers of their specific target cells and which may therefore allow dissociation of antigen-reactive cells from the antigenic stroma.

The first experiment using F_1 hybrid intermediates in the assay of migration of GVH reactive cells was in fact the same experiment as that for which the syngeneic intermediates were shown in Fig 6.1. Fig 6.2 shows the results of the parallel F_1 hybrid recipients whose bone marrow was assayed in the same strain of F_1 hybrid. A similar degree of increase of bone marrow GVH reactivity was seen in the F_1 hybrid as in the syngeneic intermediate at 2 hrs but at 12 hrs this activity had not fallen but if anything was increased still further. This result was entirely consistent with the idea of retention of antigen reactive cells at the site of their first interaction with antigen and argues in favour of

FIG 6.2

Ex 103. 350×10^6 $HO.B_2$ TDL were transferred to each member of groups of three intermediate normal rats by iv injection either 2 or 12 hrs before death. Bone marrow cell suspensions were prepared from the six major long bones of each recipient and the suspensions from each group of syngeneic or $HO.B_2$ x Pvg/C F_1 hybrid intermediates were pooled for each time period. These suspensions together with those made from matched but uninjected control groups of rats and a sample of the $HO.B_2$ TDL were then assayed for GVH activity in $HO.B_2$ x Pvg/C F_1 hybrids using the popliteal lymph node assay.

Fig 6.2 shows the dose response relationships of each population for the F_1 hybrid strain rats that had received parental strain TDL.


Ex No 103 HO B2 TDL → HO B2 x Pvg/C F1 Int assayed in HO B2 x Pvg/C F1 Hybrids
TDL
Normal F1 B.M.
12 Hr B.M.
2 Hr B.M.
Con. L.N.
2 Hr Par. B.M.
Log10 Lymph Node Weight ± S.E.
15
10
5
1
10
100
Cell Dose x 10^6

positive selection of the minority population of reactive cells. Absolute enrichment over the starting TDL population would require purification of the marrow cells to yield only parental strain lymphocytes.

Experiments were then carried out to test the antigen specificity of this retention. The results were less clear cut and several factors that make precise interpretation of the results difficult were immediately obvious. The main difficulty was knowing whether the small inherent bone marrow GVH reactivity should be taken into account. It was argued previously that injected cells and resident host cells probably do not interact to any significant degree and therefore it could be said to be reasonable to use the uninjected marrow activity as a meaningful background control. In experiments examining antigen specificity each population must be assayed in both relevant and third party F_1 hybrids. This requires therefore, that F_1 hybrid marrow cells are injected into other semi-allogeneic F_1 hybrids with the production of a mixture of background GVH and HVG responses. In this popliteal lymph node assay the peak of the HVG reaction is however much realier at 4 days (Dorsch & Roser 1974) than that of the GVH at 7 days and is also much lower on a cell for cell basis so that interference should not be too great. However, it was consistently observed that background responses of F_1A into F_1B were greater than F_1A into F_1A. Attempts to increase the sensitivity of the system by increasing the initial localization in the marrow by splenectomizing the intermediates probably did not improve the situation as the decrease by 12 hrs in syngeneic recipients was not so great as in intact recipients. Similarly irradiation of the intermediates in an attempt to recuce background activities had the unexpected result that although the total cell number removed from bones was decimated the GVH reactivity increased by a factor of ten on a cell for cell basis

resulting in exactly the same total background activity per bone.

To compare several experiments which had many differences in detail the GVH activities were transformed by using the TDL response for each experiment as a standard calibration curve. Thus using the midpoint of each experimental dose-response line (thus ignoring the differences in slope), each population's response was expressed in TDL equivalents and this number of cells was then expressed as a proportion of the initial injected dose per whole bone marrow sampled (6 long bones) after subtraction of background TDL equivalents. This rather complex transformation of the data allowed the easiest comparison of results between experiments in which the absolute GVH assay results varied considerably. It also allowed very rough comparison with quantitative data derived from labelled cell studies.

Fig 6.3 shows a representative graph of the dose response relationships of bone marrow populations in relevant and third party assays.

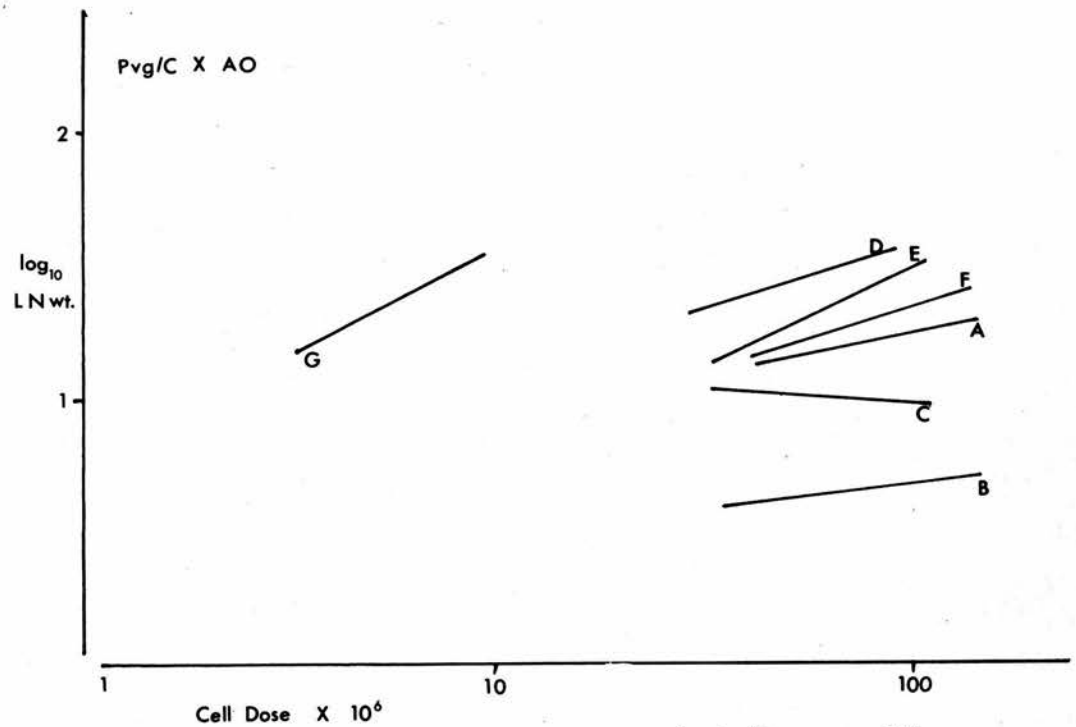
Table 6.6 summarizes the data of five experiments comprising various strain combinations and treatments of the intermediate recipients. The behaviour of parental TDL in syngeneic recipients is consistent in the three experiments in which it was measured, two of which were assayed in two types of F_1 hybrid. The results of the activities in F_1 hybrid intermediates showed a general trend towards increased retention in F_1 hybrids at 12 hrs compared to parental strain rats but the specificity of this retention was far from complete. In some strain combinations the apparent retention of GVH reactivity to third party antigens was at least as great as the reactivity to the specific antigen. There was also some variation between the degree of retention at 12 hrs compared to 2 hrs. Although this was always greater than in syngeneic

FIG 6.3

Ex 107. In this experiment triplicate groups of Pvg/C rats or Pvg/C x A0 or Pvg/C x XDA F_1 hybrid rats were splenectomized and received either no cells or 500 x 10^6 Pvg/C strain TDL 12 hrs before death. Bone marrow suspensions from these groups of animals and the original Pvg/C TDL were assayed for GVH activity in both F_1 hybrid strains.

The graphs show the dose response relationships for each population in each F_1 hybrid type.

Fig 6.3



A	Pvg/C			BM	
B	"	X	AO F1	"	
C	"	X	DA F1	"	
D	"			12 hr	BM
E	"	X	DA F1	12 hr	"
F	"	X	AO F1	12 hr	"
G	"		TDL		

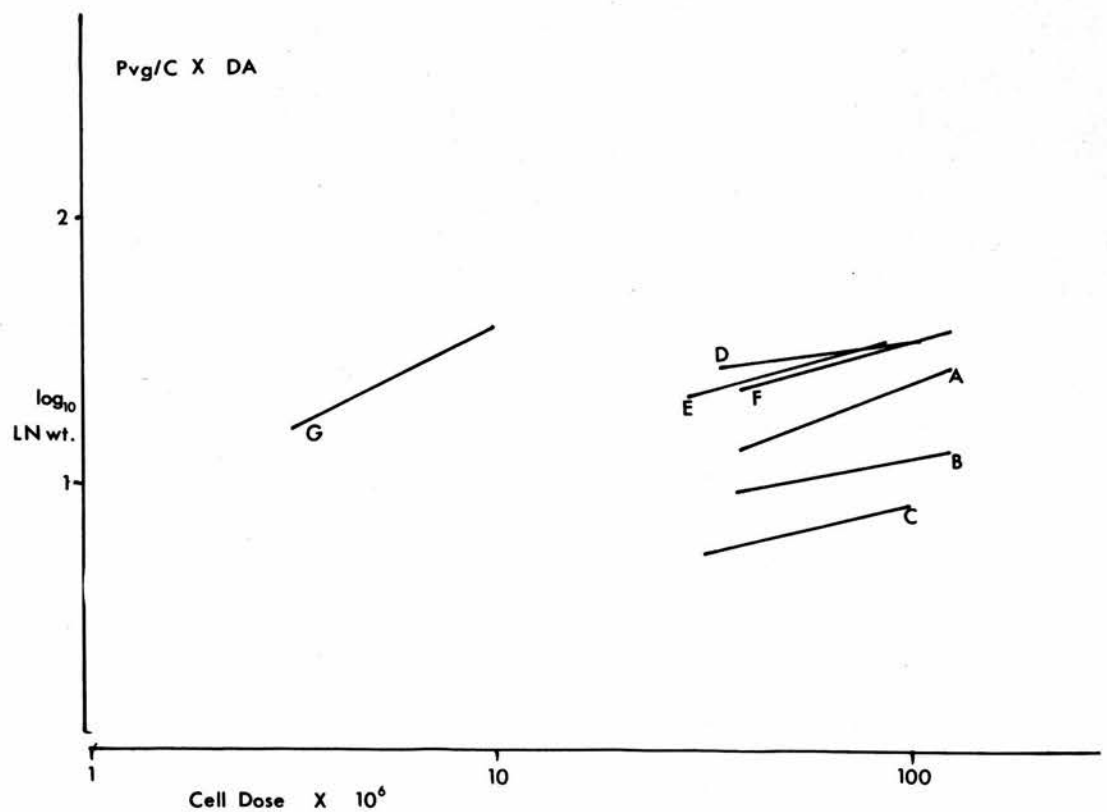


TABLE 6.6

Retention of GVH Activity in Bone Marrow expressed as percent of the Injected Cell Dose in TDL Equivalents

Experiment Number	Strain Combination and Treatment of Intermediates	2 HRS			12 HRS		
		Parental*	F_1 specific	3rd Party F_1	Parental	F_1 specific	3rd Party F
Ex 103	$HoB_2 \rightarrow HoB_2$	5.1			1.3		
	$HoB_2 \rightarrow HoB_2$ x PVG/c		5.5			11.1	
Ex 107	PVG/c → PVG/c x DA Splenectomized				1.3	7.7	9.4
	PVG/c → PVG/c x AO Splenectomized				3.3	5.7	6.7
Ex 110	PVG/c → PVG/c x DA 750 rad		2.8	6.1		3.7	2.4
	PVG/c → PVG/c x AO 750 rad		3.4	3.6		3.6	4.2

*Relative to final assay recipients.

Continued ...

Table 6.6, contd

Experiment Number	Strain Combination and Treatment of Intermediates	2 HRS			12 HRS		
		Parental*	F_1 specific	3rd Party F_1	Parental	F_1 specific	3rd Party F_1
Ex 115	PVG/c → PVG/c	9.3 8.5			1.9(2) .4		
	PVG/c → PVG/c x DA ^{51}Cr Labelled TDL injected		5.6	3.4		2.3(2)	2.4(2)
	3rd party assay in PVG/c x DA	(2) Equal activity was measured with ^{51}Cr					
Ex 121	AO → AO	4.9 3.9			.9 zero		
	AO → PVG/c x AO		4.7	3.4		1.9	.5
	3rd party assay in AO x DA						

TABLE 6.7

Tissue Distribution of Radioactivity 2 and 24 Hrs after iv Injection of ^{51}Cr Labelled Parental Strain TDL into Syngeneic or Semiallogeneic Recipients (Perfused)

% Injected Activity Per Whole Organ

2 or 3 recipients/gp. SE≏10% of mean excluded for sake of clarity

TISSUE	STRAIN COMBINATION	2 HR		24HR	
		Parental	F_1 Hybrid	Parental	F_1 Hybrid
Spleen	1	47.7	44.6	22.0	21.0
	2	45.2	50.0	22.4	22.1
	3	30.4	33.5	10.1	14.9
Bone marrow	1	22.6	26.3	$3.5^{\pm}.33$	$6.2^{\pm}$ .36
	2	15.0	13.2	2.2	2.5
	3	21.2	17.5	4.3	4.1
Lymph node	1	3.4	3.1	26.5	21.6
	2	5.4	2.9	46.5	70.0
	3	1.2	1.3	13.3	15.7
Blood leucocytes	1	1.9	1.2	1.9	1.7
	2	1.8	1.4	2.2	1.5
	3	.8	.8	1.1	1.6

Strain Combinations: (1) AO → AO or AO x DA F_1. (2) PVG/c → PVG/c or PVG/c x AO F_1.
(3) PVG/c → PVG/c or PVG/c x DA F_1.

parental controls it was not always as complete as in Ex.103 and 110. In Ex.115, ^{51}Cr labelled parental TDL were used so that an attempt could be made to calibrate the final GVH response against the radioactivity of the sample as a measure of the injected cells. In this experiment no detectable difference was seen between the radioactivity at 12 hrs in parental or F_1 hybrid intermediates.

To examine this question of absolute retention in the bone marrow of semi-allogeneic recipients kinetic distribution studies were undertaken using ^{51}Cr-labelled parental TDL in various strain combinations. The results are summarized in Table 6.7 and again only show obvious retention in bone marrow in one experiment which was incidentally the same AgB combination (AO→DA) as the ^{3}H-Udr labelled TDL experiment which had also shown 24 hr retention in F_1 marrow at about twice that in the parental strain recipients.

A final group of experiments studied the activity of bone marrow populations of syngeneic and F_1 hybrid recipients of parental strain TDL from HSA-primed donors. The aim was to investigate whether retention of GVH reactivity in an F_1 hybrid intermediate host was accompanied by retention of memory cell activity for a conventional protein antigen. The experimental design used was as previously detailed and bone marrow populations from irradiated intermediates were assayed in irradiated final parental strain rats for their ability to produce a secondary antibody response to soluble HSA.

The results are summarized in Table 6.8 which shows the mean Day 21 serum antigen binding capacity for groups of up to five assay animals which had received bone marrow cells collected 2 or 12 hrs after iv injection of primed TDL. The values of ABC are not directly comparable between experiments as the starting TDL populations had different potencies and also different proportions of the total bone marrow cells collected

TABLE 6.8

Recovery and transfer of HSA primed TDL migrating through the B M of syngeneic and F_1 hybrid intermediate rats. The day 21 ABC values are geometric means of 5 recipients each, after transfer of intermediate B M into final irradiated recipients.

Ex No.	Strain combination and treatment of intermediates	Time of B M collection after injection of HSA primed TDL	
		2hr	12hr
		ABC (± SE)*	ABC (± SE)
T95	Syngeneic parental PVG/c intermediates 800 rads	4.6 (± .15)	0.81(± .16)
T96	Semiallogeneic PVG/c x DA F_1 hybrid intermediates 800 rads	6.7 (± .08)	4.8 (± .14)
		½hr	12hr
S3 **	Syngeneic parental PVG/c intermediates 1000 rads	4.5 (± .16)	2.7 (± .34)
	Semiallogeneic PVG/c x DA F_1 hybrid intermediates 1000 rad	2.7 (± .25)	7.7 (± .10)

*Geometric mean ABC (± SE of mean $\log_{10}$ ABC).

**Tested at 0.1µg sHSA/ml.

were used.

It is again clear that in syngeneic recipients a marked fall in activity occurs between $\frac{1}{2}$ and 2 hrs and 12 hrs after iv injection. However, in the F_1 hybrid recipients this fall is much less marked or even reversed.

All the experiments using this HSA antibody assay were done in collaboration with Dr E B Bell.

Discussion

The results of the migration studies using functional cell markers in syngeneic animals confirmed the findings using radio-labelled lymphocytes and although quantitation could not be as accurate the proportions of the injected activity found in the bone marrow at the different time intervals were in the same range as measured with ^{51}Cr. In addition these functional assays showed that both recirculating T cells (GVH activity) and memory recirculating B and T cells (HSA adoptive secondary response) used this pathway. Whether B cells migrate more slowly through this tissue as has been suggested for spleen and lymph nodes (Nieuwenhuis & Ford 1976) cannot be decided from these results, as the fall in the adoptive antibody response between 2 hrs and 12 hrs could simply reflect the emigration of helper T cells specific for the antigen as these helper cells limit the size of final antibody response in this assay (Bell & Shand 1975). Thus at 12 hrs memory B cells could still be present but have insufficient 'help' to allow their full functional potential to be demonstrated.

The ability of the bone marrow environment to support lymphocyte activation by antigen with subsequent cellular proliferation was clearly demonstrated by the retention and blast transformation of parental ^{3}H-Udr labelled lymphocytes in the bone marrow of F_1 hybrid recipients whose tissues were antigenic to the injected cells.

The results of increasing localization of radiolabelled

particles in the bone marrow and in the spleen but not elsewhere suggest that macrophages lining bone marrow sinusoids are able to concentrate antigenic particles and therefore the bone marrow has both necessary prerequisites for functioning as a secondary lymphoid organ in the initiation and propagation of immune responses. A rough estimate of the likely efficiency of this site, in terms of initiation of an immune response when compared to the spleen, can be made if it is assumed that the limiting factor for immune reactions would be the likelihood of interaction between antigen reactive cells and macrophages suitably primed with antigen. If it is assumed that the marrow weight is approximately half of that of the bones containing marrow, then this organ would be ten to fifteen times as large as the spleen, yet it supports a lymphocyte flux of seven-fold less per whole organ and has a total antigen localizing capacity of only four to five times as great. Thus the total efficiency is likely to be of the order of 0.2% of that of the spleen/unit weight or 3.0%/ whole organ.

This functional capability is entirely consistent with the growing body of evidence that the bone marrow is an important site of plaque-forming cell localization, particularly in secondary responses as well as late in primary responses (Benner et al 1974a). It would also explain the observation that the bone marrow is only important in primary antibody responses in the absence of the spleen if an approximately 40 times greater antigen dose is injected iv (Benner et al 1975). The high rate of lymphocyte flux could explain the distribution of plaque-forming cells or their precursors in this site as such cells would be likely to migrate randomly from the blood into the tissues and might find the marrow an ideal site for maturation into relatively sessile plasma cells especially in the presence of antigen.

The thorough studies of Benner et al (1974 a,b & c,1975)

on the generation of memory B and T cells and plaque-forming cells in mouse bone marrow and other tissues, showed that IgM, IgG and IgA secreting cells could be detected in the bone marrow of normal animals under appropriate conditions and also showed that this site provided the majority of such cells in the body late in secondary responses. However, their conclusion that priming in splenectomized recipients after large antigen doses occurred in the mesenteric lymph node or Peyer's patches following localization of small amounts of antigen in those sites is less likely than the alternative explanation of priming in the bone marrow which they viewed with less favour.

The importance of the idea of lymphocyte flux rather than a simple seeding of peripheral organs including the bone marrow for plaque-forming cells, their precursors and associated helper T cells, is suggested by the finding of greater numbers of plaque-forming cells in the bone marrow of mice splenectomized between priming and secondary challenge with sheep erythrocytes compared to intact controls (Benner + Oudenaren 1975). In the splenectomized animals the flux of lymphocytes through all tissues is likely to be increased (Bradfield & Born 1973; this thesis Ch.4). Conversely in intact recipients the spleen would compete with the bone marrow for the recirculating B and T memory cells present in the blood.

During a primary response to sheep erythrocytes plasmablasts, the activated precursors of antibody secreting cells are produced in the periphery of the splenic periarteriolar lymphoid sheath and migrate via the marginal zone bridging channels (van Ewijk 1977). This is a normal exit pathway of recirculating lymphocytes which enter venous blood in the red pulp sinuses (Mitchell 1973). The major distribution of plaque-forming cells in the spleen is in the red pulp cords and if entry into this site entails passage via the blood rather than by direct

interstitial migration from the white pulp it is to be expected that the spleen would be in direct competition with the rest of the body and that the proportional distribution throughout the body would reflect the flux of lymphocytes through tissues as well as the distribution of antigen. This process would explain the widespread distribution of antibody secreting cells including the significant bone marrow localization late in primary immune responses to intravenous antigens that begin in the spleen. This holds for both T independent antigens such as LPS (Benner et al 1976), and thymus dependent antigens such as sheep erythrocytes (Benner et al 1974), or hen lysozyme protein (Hill 1976).

Functional studies of bone marrow cell populations also show that many lymphocyte sub-populations are represented; mature T cells:

(i) GVH reactivity (Yoshida & Osmond 1970)

(ii) adoptive primary haemolysin response to sheep erythrocytes (Howard & Scott 1972)

(iii) sheep erythrocyte memory helper cells (Benner et al 1974)

(iv) hen lysozyme memory helper cells (Hill 1976)

(v) cells protective against 1b leukaemia in C58/wm mice (Lukasewyvz et al 1976)

and immunologically active B cells:

(i) IgM, IgG, and IgA plaque-forming cells to sheep erythrocytes (Benner et al 1974a)

(ii) plaque-forming cells to LPS (Benner et al 1976)

(iii) memory precursors of plaque-forming cells to sheep erythrocytes (Benner et al 1974b)

These functional attributes are confirmed by surface markers.

The true thymic dependency of bone marrow T cells has been shown by the absence of θ-bearing cells in the bone marrow

of congenitally athymic nude mice and also by their reduced frequency in thymectomized normal mice (Claman 1974) thus indicating that such T cells enter from the blood.

Further reported observations are readily understandable if a substantial lymphocyte flux through the bone marrow is recognized. There is a net redistribution of both B and T cells to the marrow in mice (Moorhead & Claman 1972) and guinea pigs (Fauci 1975) following systemic corticosteroid administration. This phenomenon is rapid, as is the recovery following metabolism of the hormone and could follow a change in transit time of lymphocytes through this and other compartments of the recirculating pool as is suggested by the rapid decrease that also occurs in thoracic duct output of rats (Spry 1972).

Recovery of B cell numbers in irradiated fetal liver cell reconstituted mice that are also given Strontium-89 which localizes in bone and therefore continuously irradiates the neighbouring marrow, is incomplete (Rozing et al 1976, Kincade et al 1975). In this model designed to show that B cells are able to mature in a micro-environment other than the haemopoietic bone marrow, mature B cells and possibly their precursors released from the spleen into the blood are likely to migrate into the marrow and therefore a proportion may succumb to irradiation damage. If this were true, depletion of the recirculating pool should also occur in normal animals given ^{89}Sr in a way analagous to local irradiation of the spleen (Ford 1968) or extracorporeal irradiation of blood (rev. by Cronkite 1968).

After iv injection of Newcastle disease virus into rats there was acute lymphocytopenia and also depletion of cell numbers in the periarterial lymphoid sheath in the spleen and the deep cortex of lymph nodes. This situation developed quickly and the recovery 24 hrs later was also rapid without evidence of new cell production as shown by low labelling indices after ^{3}H-Tdr administration in these populations. Thus the depletion of recirculating lympho-

cytes must result from a net redistribution as could occur if tissue transit times were altered. It is significant that no excess of injected ^{51}Cr labelled cells in this situation was measured in liver or lungs and also that normal levels of label were found in the spleen even in the presence of marked lymphoid sheath depletion, whereas there was significant reduction in lymph node localization (Woodruff & Woodruff 1972). The possibility arises that the sites of net redistribution are the splenic red pulp and the bone marrow which was not examined.

The distribution of newly formed B lympbocytes which are known to migrate to secondary lymphoid organs by tracer studies using marrow cells labelled locally with ^{3}H-Tdr (Brahim & Osmond 1976) may also involve migration to other bone marrow sites. Occasional labelled cells were noted in distant marrow sites in similar experiments (Brahim & Osmond 1973) and the presence of cells in subcapsular sinuses of lymph nodes and in efferent lymph sinuses argues in favour of the migratory capacity of such cells. Thus an explanation of the apparent random emigration of B cells at different stages of maturation could be that cells are released at similar stages of relative immaturity and after more or less extramedullary maturation a proportion of the cells could re-enter marrow at random sites. This would give rise to a similar distribution pattern overall.

The apparent sparing of GVH reactivity of the bone marrow in heavily irradiated rats which is unexpected in view of the sensitivity of lymphocytes to irradiation may also be explained by a redistribution of surviving cells from the blood into the marrow. Evidence in favour of this explanation is the increased early localization of ^{51}Cr-labelled lymphocytes in bone marrow of irradiated recipients as shown earlier and also by the similar increase noted in irradiated marrow rather than control marrow in cross-transfusion experiments in which ^{3}H-cytidine labelled peripheral blood cells were counted in tissue

autoradiographs one hour after transfusion into rats (Bond et al 1964).

The experiments involving the behaviour of parental strain lymphocytes in F_1 hybrid recipients showed that lymphocytes can recognize bone marrow tissue as foreign and react by transformation and division in this site. Thus a systemic graft versus host reaction includes the bone marrow in its initial stages as well as the splenic white pulp and lymph nodes (Gowans 1962, Ford, Simmonds & Atkins 1975). The reaction in the splenic white pulp is associated with destruction of this tissue (Gowans 1962) within ten days of the injection of the parental strain lymphocytes, and the splenomegaly that is noted during the first week of the reaction is mainly a reflection of expansion of the red pulp by proliferation of host haemopoietic tissue. This would be expected if the normal haemopoietic organs were undergoing destruction as is highly probable following retention and proliferation of donor lymphocytes in the bone marrow. This could also provide some explanation of the time course of fatal graft versus host disease for it could be suggested that the animal survives until the sites of extramedullary haemopoiesis are also invaded and destroyed by cytotoxic effector lymphocytes or their precursors which would follow only after the splenic red pulp for example had enlarged to such an extent that it supported a greater flux of lymphocytes. This would include antigen reactive cells that are eventually released into the recirculating lymphocyte pool from the sites of primary proliferation (Sprent & Miller 1973, Nieuwenhuis et al 1974).

The difficulties in demonstrating specific enrichment of antigen reactive cells in the tissues of the F_1 hybrids within the first 24 hrs after initiation of a systemic GVH reaction include the possible under-representation of such cells in single cell suspensions, altered migration of transforming cells, possible refractoriness of transforming cells to further antigenic stimulation, as well as the

effect of background reactions of the tissues in the assay and the inability to quantitate the total numbers of donor type cells in the cell suspensions assayed. Attempts to clarify the situation particularly with regard to the background activity by using splenectomized or irradiated recipients as intermediates were unhelpful for the reasons already discussed. Quantitation of donor cells using ^{51}Cr labelled TDL as the initial parental population has the inherent inaccuracy of labelling not just the antigen-reactive cells and also reflects variation in the total retention of labelled cells in lymphoid tissues of semi-allogeneic recipients as described. This variability was also noted by Heslop & Hardy (1971) in various allogeneic and semi-allogeneic rat strain combinations.

A possible way of both quantitating donor type lymphocytes and also abolishing the background activity of F_1 bone marrow populations would be to treat the cell suspensions in vitro with a powerful cytotoxic alloantiserum of parental type directed against the alternative allele of the particular F_1 hybrid.

In spite of these difficulties the results of both functional and labelled cell studies suggest that there is significant retention of parental lymphocytes in F_1 hybrid bone marrow but that this retention shows variable antigen specificity. Depending upon the strain combination there was retention of activity against one AgB antigen in F_1 hybrids of supposedly unrelated AgB type. Two explanations of this phenomenon can be considered. Firstly, the reaction of transformation in antigen reactive cells could release local mediators that cause retention of third party lymphocytes that happen to be in the vicinity. The variation between strain combinations must be explained by variable liberation of such mediators. On the other hand, if the retention of antigen reactive cells is not associated with liberation of such factors it must be suggested that there could be significant cross-reaction

between cellular recognition of certain different AgB antigen complexes. The lack of cross-reactivity between such antigens as shown with serum antibodies or depletion experiments using in vivo filters and examining thoracic duct cells does not necessarily preclude such an explanation. If there was a range of avidities for each AgB antigen it may follow that specific cells of all avidities are retained in the tissues of an F_1 hybrid so that the deficit in the recirculating pool is indeed complete. If high avidity cells are disproportionately more powerful in the local GVH assay and low avidity cells for third party antigens were retained because of some cross reactivity, then the real deficit of these latter cells from the recirculating pool may not be measurable with this assay. In the tissues, however, the presence of this type of cell may be enough to show functional activity in the assay in the absence of many high avidity 3rd party reactive cells, although a few of the latter cell type would be expected by random distribution.

Serum alloantibodies may not react to the same part of the major histo-compatability complex as is important for cellular recognition and therefore may not show the same cross reactivity. The apparent non-cross reactivity of selectively enriched populations parked in B rats following mixed lymphocyte cultures (Wilson et al 1976) or fractionation using anti-idiotype antibody (Wigzell et al 1975) may be due to the selection of high avidity cells. Recently significant cross reactions have been noted in populations of rat lymphocytes reacting in sequential mixed lymphocyte reactions after which enrichment for reactivity against one AgB type is accompanied by variable reactivity to other AgB antigens (Antzac D. pers.comm.).

One possible means of testing whether non-specific retention of bystander cells occurs would be to examine the retention of A strain parental cells specifically tolerant of strain B antigen in the bone marrow of (A x B)

F_1 hybrids undergoing a GVH reaction following the simultaneous injection of normal A strain lymphocytes by assay in A x C F_1 hybrids. If non-specific retention occurred then the reaction to the third party C antigen of bone marrow cells would be expected to be as great with the combination of normal and B tolerant lymphocytes as with normal and normal lymphocytes whereas if the retention was due to cross reactivity the B tolerant cells would not be retained in the (A x B)F_1 marrow and would not therefore be available to react with the C antigen on adoptive transfer, assuming that tolerance involves functional inactivation of high + low avidity cells and that tolerant B cells have normal GVH activity against C antigens. The second assumption could be tested experimentally.

Similar difficulties arise in the interpretation of the results of localization of HSA memory TDL in the bone marrow of F_1 hybrids. The trend towards retention or even increase of memory activity at 12 hrs in F_1 hybrids as opposed to syngeneic parental controls can again be explained in two ways: either by non-specific retention of bystander cells in the F_1 hybrid marrow at the site of the GVH reaction, or by true cross-reactivity of a proportion of GVH reactive cells for the convential protein antigen.

That specifically selected T cells for one AgB antigen following mixed lymphocyte culture activation and 'parking' in 'B' rats had normal reactivity against sheep erythrocytes was reported by Herber-Katz & Wilson (1976) and it is theoretically probable that GVH reactive cells must carry specificity for at least one conventional antigen complexes that exist within a species.

It is perhaps unfortunate that the particular strain available for the HSA memory cell experiments was the same as that which showed greatest apparent non-selective retention

of GVH reactivity (in F_1 hybrid recipients).

However, the bone marrow as a tissue that has a low background of immuno-competent lymphocytes and supports a high flux of such cells, could provide a useful tool for the closer examination of problems of this type in various strain combinations especially if allo-antiserum purification of the retained cells proved effective.

Conclusions

The evidence argues strongly in favour of the bone marrow being able to act as a secondary as well as a primary lymphoid organ. This tissue supports a high lymphocyte flux, has a rich and active macrophage compartment and is able to support transformation and proliferation of antigen-activated lymphocytes. Estimates of the likely efficiency of this immune function in bone marrow in comparison to the spleen with which it shows many similarities, suggest a figure of about 2-3% of total splenic activity for intravenous antigens.

The bone marrow is also one of the sites of initial reaction during a systemic graft-versus-host reaction.

CHAPTER SEVEN

General Discussion

Lymphocytes are vitally important for the maintenance of homeostasis of complex animals with regard to the fate of a wide range of substances most of which are large molecules. The ability of lymphocytes to recognise molecular shapes by interaction with specific surface receptors and to mediate immune reactions following such antigenic stimulation has allowed a great increase in the efficiency of the inactivation or disposal of inappropriate molecules and organisms by the general mechanisms of inflammation together with phagocytosis and digestion. A major factor that allows these functions to act economically is the high mobility of individual lymphocytes, while the interaction of lymphocytes with other cells - eg macrophages, other lymphocytes and vascular endothelial cells - by either direct contact or via humoral factors no doubt increases both the efficiency and range of responses that can be generated.

The phylogenetic development of antigen recognition, cell mobility and vascular circulation systems are closely linked. In invertebrates lacking blood systems wandering protective cells - ameobocytes - are present. These cells apparently originated as part of the feeding mechanism which retained protective function when the digestive processes became fixed and external to the organism (Barnes 1968). Also many invertebrates - eg the earthworm, an annelid - have a coelom that performs some of the functions of a fluid circulation and contains a system of circulating cells and also performs some filtration functions. These coelomocytes comprise a heterogeneous mixture of cells that includes lymphocyte and phagocyte-

like morphologies and these cells have been shown to be active in the rejection of allogeneic skin grafts (Cooper 1976). In early chordates - eg lamprey and amphioxus - there is development of collections of immuno-competent cells and haemopoietic tissue associated with the supportive stromal tissue around the feeding tubes and the intestine in the larvae (Young 1962); again a position likely to be exposed to high concentrations of antigen. These collections contain phagocytic and lymphocyte-like cells and this tissue can be viewed as a forerunner of the spleen which develops haemopoietic, lymphopoietic and phagocytic filtration functions. In higher vertebrates the first organized lymphoid tissue to appear in ontogeny is the thymus which develops as an epithelial outpouching of the pharynx - again part of the intestinal tract (Rowlands 1976). This epithelial structure is soon invaded by lymphocytes which presumably originate in haemopoietic tissue in the yolk sac or foetal liver. As the thymus develops reticular macrophages are also included in the inductive stroma of the gland.

Thus protective lymphocytes and macrophages as mobile intestitial cells antedate the organization of lymphoid tissues or circulatory systems of either blood vessels or lymph channels. However, the appearance of blood circulations in vertebrates is associated with the development of mesodermal structures that produce circulating cells - haemopoietic tissue which includes lymphopoiesis and this type of tissue is closely associated with a stroma comprising reticular macrophages, phagocytic macrophages and endothelial cells. Thus haemopoietic tissue is usually associated with a filtration function and production of immuno-competent cells, and thus the immune, haemopoietic, circulatory and reticulo-endothelial systems are intimately interwoven.

As suggested in the introduction immune responses are the result of interaction of antigen, macrophages and lympho-

cytes, all of which may be mobile and this mobility is largely affected by the particular vascular arrangements of a tissue and also by the type and state of the endothelium of such vessels. It might be expected from the phylogenetic evidence that lymphocytes would be able to migrate to most tissues and it is unlikely that this function should be lost with the development of the very high migratory flux through specialized secondary lymphoid organs - the spleen - and in mammals the lymph nodes. However, it was in these organs that this innate migratory ability of lymphocytes was first recognized and therefore it is not surprising that migration through other tissues which occurs at much lower concentrations was considered to be likely to be less important.

The experiments reported here were designed in the conceptual framework of the recirculating lymphocyte pool as described by Gowans and his colleagues, but the number of potential compartments was extended to include all tissue types. The results were consistent with the basic assumptions involved in this model system but the experiments were not really designed to test these assumptions in any new way.

Thus in the rat, thoracic duct lymphocytes which are naturally destined for the central venous circulation, are distributed to the tissues at random with the blood. The rate of extravasation into each tissue can be experssed as a clearance factor which can be defined as the proportion of total lymphocytes presented to a unit weight of tissue that is cleared from the blood on a single passage. Thus total extravasation will depend upon: the blood concentration, the blood flow, and the microvascular architecture, as well as the nature of the vascular endothelium. The total flux through a tissue will also depend upon the interstitial route, distance and mode of exit traversed by the lymphocytes as these factors will all influence the transit time.

The evidence suggested that the flux of lymphocytes through normal non-lymphoid tissues was significant and that though the clearance rate was low the interstitial transit was rapid. It was suggested that this transit time may be as short as two or three hours which results in a much faster flux than that through lymph nodes in which a modal transit time for T lymphocytes of eighteen hours is well documented (Ford & Simmonds 1972).

The vascular endothelium thus acts as a barrier through which blood lymphocytes have to pass to enter any tissue. Most lymphocyte emigration occurs in small post-capillary venules. Evidence for this has come from (i) early localization of radio-labelled cells by autoradiography (Gowans & Knight 1964, Goldschneider & McGregor 1968) and (ii) light and electron microscopy of inflammatory lesions (Astrom et al 1968).

The post-capillary venules in lymph nodes and Peyer's Patches are morphologically specialised, and these particular vessels support a very high clearance rate and are highly selective for lymphocytes. It has been estimated that as many as 20% of blood lymphocytes entering a lymph node in the blood emigrate to the efferent lymph (Hay & Hobbs 1977). This figure was derived from estimates of blood flow by ^{89}Sr particle embolization, blood lymphocyte concentration and efferent lymphatic lymphocyte content. Similar values of blood flow to lymph nodes in the rabbit have been reported (Herman et al 1976). The computation did not take into account the possible contribution of the afferent input to the sheep lymph node which might lower the overall clearance factor but this factor would be counteracted by the fact that the total blood flow would overestimate the blood flow to the post-capillary venules which are restricted to the paracortex.

Other vascular networks, which support such a high extravasation of lymphocytes, are post-capillary in position, but are not morphologically specialized, are the marginal sinus

of the spleen (Gall 1950, Dubreuil et al 1975) and to a slightly lesser extent the sinuses of the bone marrow and possibly the liver. It is also significant to note that the differential effect on the localization of trypsin-treated lymphocytes follows the same pattern suggesting that the morphological specialization that increases the flux through lymph node blood vessels is intrinsically different from the endothelial state of spleen sinuses. As already argued, the similar trypsin sensitivity that was seen in lymph nodes and cell-mediated immune inflammatory lesions in which no morphological change was seen, suggested that lymph nodes which are phylogenetically very recent may have arisen as an adaptation that followed the maintenance of a qualitative change in venular endothelium that occurred as a temporary change in inflammatory lesions. In other words the similarities observed in structure and kinetics of lymphocyte migration through chronic granulomata (Smith, McIntosh & Morris 1970b) to those through lymph nodes can be viewed as suggesting that the lymph node acts as an analogue of the granulomatous reaction rather than vice versa.

In consideration of the ability or efficiency of any tissue to be able to support the generation of an immune response itself the factors already mentioned must be taken into account. Thus the lymphocyte flux dependent upon the lymphocyte-endothelial interaction will control the concentration of antigen reactive cells, while the concentration of antigen and particularly that made more immunogenic by processing, will depend upon the macrophage content of the tissue.

In non-lymphoid tissues the antigen concentration may be high but the likelihood of sensitization of the minority of specifically reactive lymphocytes (contained in the 10^4 - 10^5 cells that extravasate per gram each hour) being important in the total response is low unless no antigen reaches the draining lymph nodes in which relevant inter-

actions are many orders of magnitude more likely. However, in the efferent arm of immune responses the peripheral extravasation of specifically reactive cells must be of importance in the triggering of inflammation.

The observations relating to lymphocyte flux through the so called primary lymphoid organs suggested that particularly in the bone marrow a substantial proportion of blood lymphocytes entered the marrow parenchyma. This flux could be explained in terms of the normal immune function of lymphocytes rather than necessitating the suggestion of some novel function of lymphocytes traversing an area of rapid and extensive cellular proliferation. The evidence of significant antigen concentration in bone marrow and the ability of lymphocytes to undergo antigen stimulation, blast transformation and mitotic division in this environment in an antigenic F_1 hybrid host suggested that the bone marrow could act as a secondary lymphoid organ.

This similarity between type of lymphocyte flux and extent of antigenic concentration in spleen and bone marrow can be regarded as only part of a much more extensive similarity of structure and function of these two tissues. If the microanatomy of the circulation to the two organs is considered then both have similar arrangements in that the major nutrient arteries branch acutely to give rise to arterioles. In the bone marrow of mice the radial arterioles penetrate the bone and give rise to a capillary network. This network doubles back and drains into the superficial venular sinuses of the marrow space which in turn drain centrally to larger venous sinuses (de Bruyn 1970).

In the spleen the central arterioles of the white pulp give rise to penicillar arterioles which supply capillaries to the white pulp and then empty into the marginal sinus and then the red pulp venous sinuses. In both tissues there are some direct connections between arterioles and the larger venous sinuses (Dubreuil et al 1975).In addition

both tissues can support the full range of haemopoiesis including non-antigen driven lymphopoiesis and it is interesting to note that although the splenic lymphocytes are clearly concentrated around the central arterioles in the lymphoid sheath a similar anatomical localization of lymphocytes around the distributive arterioles in the bone marrow parenchyma has also been noted in the rat (Weiss 1976).

There is thus considerable overlap between the functions of different organs. The bone marrow and spleen in adult rodents can be viewed as two organs of haemopoiesis, blood filtration and immune function that show different degrees of efficiency for each aspect. Also it has been noted in the embryo of the dog and marmosa (a marsupial) in the mesenteric lymph node as it develops in the lymph spaces around the mesenteric artery that some granulopoiesis occurs before any concentration of lymphocytes and that the arrival of large numbers of lymphocytes is correlated with the development of 'specialized' post-capillary venules (Bryant & Shifrine 1972, Bryant 1974).

The other primary lymphoid organ is the thymus which has a complex anatomy. The cortex which is a site of non-antigen driven lymphopoiesis is supplied by a capillary network arising from nutrient arterioles in the medulla. here are very few post-capillary venules in the cortex as the capillaries drain into subcapsular surface venules (Blau 1977). This may in part explain the very low lymphocyte extravasation rate in the thymic cortex. The vascular arrangement in the medulla has been less clearly described but it is in this site that lymphocytes extravasate, that macrophages that can localize antigen are found, that germinal centre formation can occur, and also that morphological specialization of venular endothelium can be found under pathological conditions - eg myasthenia gravis (Bradfield 1973). Thus again at least the thymic medulla has some secondary lymphoid function under some

circumstances and this idea is supported by the similarity of lymphocyte migration kinetics in the thymus and lymph nodes.

It is thus crucial in the interpretation of in vivo immunological reactions and lymphocyte function to avoid the notion that one particular organ has an exclusive function within the system. The other important consideration to remember is the mobility of lymphocytes such that the likelihood of any lymphocyte found in a particular compartment having been in that situation for longer than a few minutes or hours is very low.

Most of the evidence suggests that lymphocytes are distributed with the blood mainly at random extravasating when and where they have the chance. The segregation of subpopulations either by type, antigen specificity, or tissue of origin, appear to occur extravascularly. The latter phenomenon is suggested by the different tissue distributions of similarly treated populations of lymphocytes collected from anatomically distinct efferent lymphatic vessels - eg peripheral somatic lymph noces as opposed to mesenteric lymph nodes. Changes have been noted with ^{51}Cr labelled whole populations (Scollay et al 1976) and small T lymphocytes (Cahill et al 1977) but are more obvious in the lymphoblast populations labelled with ^{125}I Udr (Hall et al 1977, Rose et al 1976). Although differences are noted in the lymph node localizations it is feasible that all of this difference can be accounted for by differential extravasation at the peripheral tissue level. This is perhaps a more likely site for critical differentiation as the overall clearance rates are lower than in the lymph nodes. However, rigorous testing of any selection by any criterion occurring at the level of the endothelium, either in lymph node or in peripheral tissues, would require high cell specific activity labelling and critical examination of tissue distributions at very early time intervals, as the high flux rate and low clearance factors in most tissues would make inter-

pretation of distribution data at later time intervals very difficult.

The same random processes affecting lymphocyte distribution are probably active in inflammatory lesions but the precise site of interaction of antigen and lymphocyte that allows the endothelial change which in turn allows a greater clearance of blood lymphocytes, remains a mystery. Presumably soluble factors released from lymphocytes may be involved in influencing the vascular changes and it is interesting to note that the increased blood flow, permeability and extravasation of blood leucocytes in short term acute responses to such agents as carageenin injected into rat footpads is abolished in leukopenic rats but the response is largely restored by injecting washed lymphocytes twenty minutes before the test injection (Bechara et al 1976). This rapid response in normal rats is unlikely to be mediated by a specific immune response and suggests rather some non-specific effect on the normal endothelium of varying concentrations of circulating lymphocytes or their products in the blood. If this effect is confirmed then it will be even more important to investigate further the mechanism of the inter-relationship between endothelium and lymphocytes.

The important conclusions of the experiments reported in this thesis include the confirmation of significant migration of lymphocytes through all non-lymphoid tissues which are therefore compartments in the recirculating lymphocyte pool of rodents. This migratory flux is enhanced by inflammation and at least in the case of cell mediated immune responses this is the result of a qualitative change in the lymphocyte-endothelial interaction as shown by the effect of trypsin-treatment of injected lymphocytes. The substantial migration of lymphocytes through the so-called primary lymphoid organs indicates the immune function of these organs is not solely the production and maturation of precursor cells. This may explain many

observations relating to the development of immune responses and life history of lymphocytes in the whole animal.

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Appendix

Published papers which include data presented in this thesis.

Rannie G H, Smith M E and Ford W L
Nature 267 520-522, 1977.
Lymphocyte migration into cell-mediated immune lesions is inhibited by trypsin.

Rannie G H and Donald K J
Cell Tissue Kinet. 10 523-541, 1977.
Estimation of the migration of thoracic duct lymphocytes to non-lymphoid tissues. A comparison of the distribution of radioactivity at intervals following iv transfusion of cells labelled with ^{3}H, ^{14}C, ^{75}SE, ^{99m}Tc, ^{125}I and ^{51}Cr in the rat.

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Physiology of lymphocyte recirculation in animal models.

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Lymphocyte migration into cell-mediated immune lesions is inhibited by trypsin

THE lymph nodes and the white pulp of the spleen are major sites into which lymphocytes migrate from the blood and each organ is equally selective in that other blood cells are efficiently excluded[1,2]. It might be expected that the same mechanism for discriminating between lymphocytes and other cells would have evolved in the spleen and lymph nodes, but the entry of lymphocytes into each of these organs can be distinguished in two ways. First, the histological appearance of the vascular endothelium across which lymphocytes migrate is dissimilar[1,3] and second, the capacity of lymphocytes to migrate from the blood into lymph nodes is reduced by brief exposure *in vitro* to low concentrations of trypsin, but migration of the same population into the spleen is barely affected[4]. Lymphoctyes are not irreversibly damaged by trypsin because they regain their capacity both *in vivo* and *in vitro*[5] to localise in lymph nodes. The reduced numbers in lymph nodes is not a consequence of increased localisation of trypsinised lymphocytes elsewhere, as is testified by perfusing trypsinised lymphocytes through an isolated lymph node[6] and also by measuring the numbers of trypsinised lymphocytes in the blood after intravenous injection[6,7]. For several hours the concentration of treated cells in the blood was about 1.4 times that of control cells. This excess was attributed to the failure of trypsinised cells to enter lymph nodes[6]. We have extended these observations by comparing the capacity of trypsinised and control lymphocytes to migrate from the blood into several non-lymphoid tissues including sites of inflammation. Exposure of lymphocytes to trypsin not only exerts a differential effect on lymph node and spleen localisation, it also differentiates the mechanism by which lymphocytes migrate in small numbers into normal skin and other non-lymphoid tissues (trypsin resistant) from that responsible for the migration of increased numbers of lymphocytes into cell-mediated immune (CMI) lesions in the skin (trypsin sensitive).

To determine whether the differential effect of trypsin on lymph-node and spleen localisation is merely a quantitative effect, lymphocytes obtained by thoracic duct cannulation[8] of AO rats were labelled *in vitro* with either ^{3}H-uridine or ^{14}C-uridine[8]. The ^{3}H-labelled lymphocytes were exposed *in vitro* to graded doses of trypsin in phosphate-buffered saline (PBS), washed, combined with the ^{14}C-labelled cells as a reference population and injected into syngeneic recipients. No reduction of spleen localisation was found with a concentration of trypsin 125 times greater than that needed to inhibit lymph-node localisation by 50% (Table 1). The standard concentration of trypsin previously used (0.02 mg ml^{-1}) (refs 4 and 6) reduced lymph-node localisation to 10% of untreated cells at 1 h after injection and increasing the dose by 5- or 25-fold had little additional effect on either the deficit in the lymph nodes or the excess in the blood. The distribution of trypsinised cells at 24 h resembled that of control cells and only at the highest concentrations was there evidence of loss of a minority of the cells. There thus seems to be a qualitative difference in the trypsin sensitivity of lymphocyte migration into the spleen and lymph nodes.

Lymphocytes also migrate from the blood into non-lymphoid tissues such as skin, albeit in much smaller numbers than into secondary lymphoid organs and the rate of migration is increased into sites of CMI (refs 9–12).

Table 1 Effect of graded concentrations of trypsin on lymphocyte localisation in organs of recipients*

Ratios of ^{3}H (associated with trypsinised cells) to ^{14}C (control cells)†

Time after injection	Organ	Concentration of trypsin (mg ml^{-1}) 0.5	0.1	0.02	0.004
	Lymph nodes	0.07	0.07	0.10	0.51
	Spleen	0.99	0.96	1.02	1.02
	Blood	1.47	1.46	1.32	1.17
1 h	Lung	1.68	1.04	1.12	0.98
	Liver	1.35	1.22	1.01	0.86
	Gut	0.72	0.91	1.02	0.96
	Bone marrow	0.78	0.97	1.07	0.98
	Lymph nodes	0.72	0.76	0.89	0.89
	Spleen	1.18	1.06	1.05	0.92
	Blood	0.69	0.75	0.82	0.92
24 h	Lung	0.82	0.88	0.88	0.95
	Liver	1.15	0.91	1.07	1.04
	Gut	0.86	0.78	1.01	0.94
	Bone marrow	0.79	0.84	0.88	0.78

*Lymphocytes exposed to trypsin *in vitro* for 10 min.

†All ratios are standardised so that ^{3}H/^{14}C ratio in the injection sample = 1.00. A supplementary group of recipients received ^{3}H-labelled cells (untreated) and ^{14}C-labelled cells (untreated). As expected the ^{3}H/^{14}C ratio generally fell in the range of 0.90–1.10 indicating an even distribution. In the Table, a ratio of less than 0.80 indicates a significant deficit of trypsinised cells and a ratio of more than 1.20 a significant surplus.

We investigated whether migration into non-lymphoid tissue resembled migration into lymph nodes in being trypsin sensitive and whether trypsin treatment could distinguish between migration into normal and inflamed skin.

Lymphocytes were obtained by thoracic duct cannulation of inbred AS or (PVG/c×DA)F$_1$ rats[8]. They were radioactively labelled *in vitro* by incubation with ^{51}Cr-sodium chromate at 10 μCi ml^{-1}. The cells were suspended in medium RPMI 1640 with 10% FCS at 37 °C for 1 h. After washing, half the suspension was treated with trypsin (Boehringer) at 0.02 mg ml^{-1} for 10 min. After further washing, trypsinised and control cells were injected intravenously into separate syngeneic recipients, which were killed at 0.5, 2 or 24 h after injection. A 2-ml sample of blood was taken and the entire vasculature was perfused through an aortic cannula with a large volume of PBS. The use of ^{51}Cr as a radioactive label for lymphocytes and the perfusion procedure have been found to be the most satisfactory method of measuring lymphocyte migration into non-lymphoid tissues[13]. The spleen and the six largest superficial cervical lymph nodes were removed and also samples taken of the tissues referred to in Table 2. The specimens were weighed before radioassay in a LKB γ-counter.

Two series of experiments were performed involving recipients with contact sensitivity lesions and adjuvant granulomata. Contact sensitivity was induced by painting an area of ventral abdominal skin with 0.1 ml of 5% DNCB in alcohol. Twelve days later a site on the back was painted with a challenge dose of DNCB (0.1 ml of 0.5%). As control sites, primary application of two other contact sensitising agents, TNCB (0.1 ml of 1%) and oxazolone (0.1 ml of 0.5%), and croton oil (0.1 ml of 10%) were applied simultaneously to different areas of dorsal skin 24 h before the injection of labelled lymphocytes. In other recipients

Table 2 Effect of trypsin on ^{51}Cr-labelled lymphocyte localisation in contact sensitivity lesions
% injected activity per g tissue ± s.e.

	0.5 h $n = 7$		2 h $n = 4$		24 h $n = 4$	
	Control	Trypsin	Control	Trypsin	Control	Trypsin
Normal skin	0.029±0.004*	0.032±0.004‡	0.013±0.003	0.013±0.002	0.014±0.001	0.011±0.001
DNCB′ ($n = 16$)	0.044±0.003†	0.034±0.002§	0.032±0.011	0.020±0.003	0.052±0.015	0.041±0.010
Croton oil	0.028±0.005	0.027±0.005	0.018±0.004	0.018±0.004	0.021±0.003	0.021±0.004
TNCB	0.020±0.001	0.020±0.003	0.012±0.002	0.011±0.001	0.012±0.002	0.012±0.002
Oxazolone	0.024±0.005	0.023±0.002	0.012±0.002	0.014±0.003	0.014±0.002	0.013±0.001
Blood leukocytes	0.41±0.09	0.51±0.08	0.07±0.007	0.15±0.03	0.14±0.01	0.14±0.01
Lymph nodes	11.3±3.11	0.11±0.01	16.8±4.4	0.35±0.08	58.2±8.7	51.6±7.6
Spleen	82.1±5.6	104.7±7.9	94.6±7.9	139.6±8.2	50.8±2.7	52.5±2.1
Liver	2.1±0.33	2.8±0.45	1.2±0.18	1.5±0.07	1.2±0.05	1.3±0.05
Muscle	0.013±0.002	0.019±0.002	0.006±0.001	0.008±0.002	0.006±0.001	0.004±0.001
Bone marrow	3.5±0.35	2.5±0.33	3.7±0.25	2.5±0.05	0.88±0.11	0.69±0.12

*Against †, $P < 0.05$; † against §, $P < 0.02$; ‡ against §, not significant by the Mann–Whitney U test.

adjuvant granulomata had been produced by intradermal injection 21 d previously of 0.1 ml of Freund's complete adjuvant (Difco) emulsified with PBS.

The localisation of lymphocytes in normal skin was clearly not impaired by trypsin; radioactivity associated with trypsinised lymphocytes found in the skin soon after injection was equal to or slightly greater than radioactivity associated with untreated cells (Tables 2, 3) possibly reflecting the marginally higher numbers in the blood. It was confirmed that the migration of lymphocytes into the spleen and liver is unimpaired by trypsin treatment especially when compared with the blood level. Similarly, migration into muscle (Table 2), brain and ovary were almost equal for trypsinised and control cells suggesting that migration of lymphocytes into non-lymphoid tissues in general may be trypsin resistant. By contrast, migration into lymph nodes was reduced by trypsin to 1–2% of control values at 30 min or 2 h after injection (Table 2).

The most striking result was that trypsin treatment impaired the capacity of lymphocytes to migrate into sites of CMI in the skin (Tables 2 and 3). In the case of the DNCB lesion, the early localisation (0.5 h, 2 h) of untreated lymphocytes was increased to 1.5–2.5 times that of normal skin or sites painted with TNCB or oxazolone (Table 2). Although small, this increase was significant and occurred consistently in five other series of experiments on the migration of lymphocytes into DNCB lesions. The localisation of trypsinised cells was significantly less than that of control cells in DNCB lesions and was nearly the same as in normal skin. Thus, if it is acceptable to regard the values for normal skin as a baseline, the data indicate that very few if any trypsinised lymphocytes entered the skin as a result of DCNB challenge. The results followed the same pattern for the granuloma (Table 3) and were more emphatic: 2–3 times as many untreated lymphocytes were present in the lesions as were present in normal skin at 30 min and 2 h but equal numbers of trypsinised lymphocytes were present in the granuloma and in normal skin at each of the early intervals.

We conclude that mechanisms of selective lymphocyte migration can be placed into two categories. The first, which is trypsin resistant, operates in the spleen and non-lymphoid tissues and the second is trypsin sensitive and operates in lymph nodes and sites of CMI. Non-immune chronic inflammatory lesions have not been investigated.

It is possible that trypsin distinguishes two subsets of thoracic duct lymphocytes rather than two mechanisms of selective migration which are properties of different endothelia. In particular, could the inhibitory effect of trypsin on migration into sites of CMI be exerted solely on large lymphocytes which are known to migrate especially well into sites of inflammation[10–12]? The present results do not completely exclude this but we feel it is likely that trypsin does impair small lymphocyte migration into CMI lesions for two reasons. First, small lymphocytes do migrate into CMI lesions in increased numbers as has been supported by cannulation of the lymphatic draining an adjuvant granuloma in sheep[9] and selectively labelling small lymphocytes with ^{3}H-thymidine *in vivo* and detecting an increased localisation in DNCB lesions (G.H.R., unpublished). The effect of trypsin in preventing the increased localisation in CMI lesions was complete within the limits of detection (Tables 2, 3) and so the migration of both small and large lymphocytes was probably impaired. Second, large lymphocytes not only migrate preferentially into inflamed tissues but seem to be superior in migrating into non-lymphoid tissues in physiological conditions. This was suggested by injecting alternately labelled large and small lymphocytes and examining the cells migrating into normal rat skin[13] and non-inflamed peritoneal cavity (O. Braendstrup and O. Werdelin, unpublished). In conclusion, although it is possible that trypsin may affect lymphocyte subsets differentially, it seems improbable that this factor could wholly explain the present results; at least some lymphocytes are capable of leaving the blood by either of two mechanisms.

In an adjuvant granuloma in sheep, the increased flux of lymphocytes was associated with a change in the appearance

Table 3 Effect of trypsin on ^{51}Cr-labelled lymphocyte localisation in adjuvant granuloma
% injected activity per g ± s.e.

	0.5 h ($n = 2$)		2 h ($n = 2$)		24 h ($n = 2$)	
	Control	Trypsin	Control	Trypsin	Control	Trypsin
Normal skin	0.027±0.006	0.037±0.001	0.021±0.005	0.025±0.002	0.020±0.002	0.016±0.002
Granuloma ($n = 8$)	0.069±0.004*	0.038±0.002†	0.039±0.002‡	0.021±0.001§	0.27±0.02	0.10±0.01
Blood leukocytes	0.42±0.19	0.69±0.08	0.10±0.02	0.12±0.003	0.21±0.015	0.10±0.034

Other tissues similar to Table 2.
*Against †, $P < 0.002$; ‡ against §, $P < 0.002$ by the Mann–Whitney U test.

of the endothelium of small venules, which came to resemble the morphologically distinct high-walled endothelium in lymph nodes[9]. This is consistent with the notion that a granuloma accepts an increased influx of lymphocytes by the same mechanism as lymph nodes accept more lymphocytes from the blood than do non-lymphoid tissues. Of the two examples of a trypsin-sensitive mechanism of selective lymphocyte migration, the endothelial change in CMI may have appeared earlier in evolution since histologically well developed lymph nodes are confined to mammals. Possibly lymph nodes developed when the endothelium of small vessels in certain regions displayed as a permanent feature an adaptation which was previously unique to sites of chronic inflammation. Other features of lymph node organisation may have ensued as a result of the vascular change.

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ESTIMATION OF THE MIGRATION OF THORACIC DUCT LYMPHOCYTES TO NON-LYMPHOID TISSUES

A COMPARISON OF THE DISTRIBUTION OF RADIOACTIVITY AT INTERVALS FOLLOWING I.V. TRANSFUSION OF CELLS LABELLED WITH ^{3}H, ^{14}C, ^{75}Se, ^{99m}Tc, ^{125}I AND ^{51}Cr IN THE RAT

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ABSTRACT

The distribution of radioisotopes in tissues was measured following i.v. injection of labelled thoracic duct lymphocytes into syngeneic rats. The rate of elution of an isotope from the labelled cells and the subsequent fate of the eluted isotope were shown to be the most important factors limiting the usefulness of such isotopes for measuring cell localization particularly in non-lymphoid tissues. Comparison of labelling procedures using [^{3}H] and [^{14}C]uridine, [^{3}H] and [^{14}C]leucine, [^{75}Se]-L-selenomethionine, [^{99m}Tc]sodium pertechnetate and [^{51}Cr]sodium chromate *in vitro* and [^{3}H]thymidine *in vivo* showed that ^{51}Cr had the fewest disadvantages in the present context. Using ^{51}Cr-labelled cells, the radioactivity was measured in a wide range of non-lymphoid tissues, and estimates of cell traffic were obtained. In skin, for example, the results indicate a cell flux in the range of 10^{4}–10^{5} lymphocytes/gm/hr. Evidence is presented which suggests that the early substantial localization of labelled cells in the lung is not an artefact due to sequestration or embolization of traumatized cells but probably reflects a slow intravascular transit time through this capillary bed. The primary lymphoid organs, thymus and bone marrow were shown to include a subpopulation of lymphocytes which belong to the recirculating pool. The thymus always contained a greater concentration of radioactivity at 24 hr than all non-lymphoid tissues except liver and kidney (approx. 0·1% of the recirculating lymphocyte pool) and the bone marrow was capable of temporarily accepting a substantial proportion (approx. 25%) of the injected cells.

The substantial recirculation of lymphocytes from blood into lymphoid tissue and back to blood either via central lymph from lymph nodes or in the case of the spleen directly into the

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venous effluent is well documented (Gowans, 1959; Ford, 1969). Lymphocytes also pass from the blood into the interstices of most, if not all, tissues from where they return to the lymph nodes via afferent lymphatics and eventually to the blood in efferent lymph. Much less information is available on the quantity of such cell traffic but the low cell numbers in hourly collections of afferent lymph (Smith, McIntosh & Morris, 1970a) suggest that it is of low magnitude compared with the major recirculation pathways mentioned.

This paper presents estimates of such cell traffic to non-lymphoid tissues obtained by measuring the tissue distribution of transfused thoracic duct lymphocytes (TDLs), labelled with various radioisotopes, at several time intervals. To conclude that tissue radioactivity is attributable to cells requires close attention to the behaviour of the isotope after cell labelling and these considerations are particularly important in areas of expected low cell concentration. The latter low cell concentration precludes quantitation by autoradiographic histology which might be considered the ideal method as the presence of cells can be confirmed positively.

MATERIALS AND METHODS

Animals. Male and female rats of several highly inbred strains and their F_1 hybrid offspring weighing between 100 g and 250 g were used as donors and recipients of lymphocytes. All cell transfers were between syngeneic animals. The strains used were AO; PVG/c; DA; (PVG/c × DA)F_1 and all were bred and maintained in this department.

Isotopes. All isotopes were obtained from The Radiochemical Centre, Amersham, England except [^{75}Se]-L-selenomethionine which was supplied by C.I.S.* The preparations used are shown in Table 1.

Operative procedures. Thoracic duct cannulation was performed under ether anaesthesia by Gowans's modification of Bollman's method using polythene tubing (PP 50–Portex Plastics Ltd.). The rats were maintained and lymphocytes were collected into ice-cold flasks as described by Ford & Hunt, (in *Handbook of Immunology,* ed. by D. M. Weir, 1973). Splenectomy was carried out via a left subcostal incision under ether anaesthesia either

TABLE 1. Isotopes used

Isotope	Specific activity	Manufacturers code no.	Emission	Concentration used for labelling
L-[4,5-^{3}H]leucine	55 Ci/mmol	TRK 170	β	10 μCi/ml
L-[U-^{14}C]leucine	270 mCi/mmol	CFB 67	β	1 μCi/ml
[5-^{3}H]uridine	25 Ci/mmol	TRA 178	β	10 μCi/ml
[2-^{14}C]uridine	62 mCi/mmol	CFA 315	β	1 μCi/ml
[6-^{3}H]thymidine	5 Ci/mmol	TRA 61	β	1 μCi/g body wt/day
[5-I^{125}]iodo-2-deoxyuridine	100 mCi/mg	IM 352	γ	0·5–1 μCi/ml
Sodium[^{51}Cr]chromate	50–400 mCi/mg Cr	CJS 1P	γ	10 μCi/ml
Sodium [^{99m}Tc]pertechnetate from technetium-99m sterile generator		MCC 3	γ	up to 10 mCi/ml
[^{75}Se]-L-selenomethionine	70 mCi/mg	SEMM-1	γ	3.3 μCi/ml

* Via their British agent Eurotope Services Ltd, 104 East Barnet Road, New Barnet, Hertfordshire EN4 8RE.

immediately or 10 days before cell transfer.

In vivo labelling. Radioactive label was confined to an age-cohort of relatively long-lived small lymphocytes by administering [^{3}H]thymidine at 1 μCi/g body wt/day intraperitoneally daily for three weeks and then allowing a lapse of three weeks after the last injection before collecting TDLs.

Cell preparation. TDLs, collected for periods of up to 24 hr in duration, up to 72 hr after cannulation, were centrifuged at 350 *g* for 10 min and resuspended in medium RPMI 1640 with L-glutamine and 10% Foetal Calf Serum (F.C.S.)–(GIBCO-BIOCULT, Glasgow, Scotland) at 50–100 $\times$ 10^6 cells/ml for *in vitro* labelling.

Removal of contaminating red blood cells, even if the proportion was very low, was found to be essential when using sodium [^{51}Cr]chromate as this isotope labels all cell types. This was most successfully achieved by layering TDLs (30 $\times$ 10^6/ml) in PBS* onto 5·0 ml of a mixture of Ficoll and Hypaque (Ficoll–Pharmacia Fine Chemicals, Uppsala, Sweden. 50 ml of 14% plus 20 ml Hypaque–45% sodium diatrizoate, Winthrop Laboratories, Surbiton upon Thames, Surrey), and centrifuging at 1200 *g* for 20 min at 15°C. The interface layer was washed once in PBS by centrifuging for 10 min at 350 *g* and the resulting lymphocyte pellet was resuspended in medium for labelling as before. The yield of lymphocytes after this treatment was usually over 80% with no reduction in the high proportion of viable cells as assessed by Trypan blue exclusion.

Cells were labelled with isotopes at the appropriate concentrations for 1 hr at 37°C in a gently shaking waterbath, and washed three times in 50 ml volumes of PBS. The cells were finally centrifuged through 5 ml of 50% FCS in PBS and resuspended in medium at concentrations appropriate for injection. Cells which had been labelled by *in vivo* administration of isotope were collected from the thoracic duct, washed once in PBS and resuspended for injection.

A population of cells of accredited recirculating ability containing few labelled blast cells was obtained by passaging labelled cells from blood to lymph in an intermediate syngeneic rat with an indwelling thoracic duct cannula. Cells were collected between 6 and 24 hr after intravenous (i.v.) injection (referred to as passaged TDL).

Cell doses ranging from 10 to 1000 $\times$ 10^6 lymphocytes per recipient did not alter the proportional distribution of label. Most recipients received approximately 100 $\times$ 10^6 cells in a volume of 2·0 ml via the lateral tail vein under light ether anaesthesia.

Processing of tissues. At timed intervals after injection of labelled cells, recipients were killed and total body perfusion was performed. This procedure was designed to remove intravascular cells and it also provided useful information about the leakage of isotope from lymphocytes into extracellular fluid. Under deep ether anaesthesia and after a cardiac blood sample had been obtained, the chest was opened and a polythene tube (PP 90, Portex Ltd.) inserted through the apex of the left ventricle into the root of the aorta where it was sutured in place. Approximately 250 ml of PBS with heparin (10 units/ml) to prevent clotting was then infused either by gravity feed or by syringe. The inferior vena cava in the thorax, and the right and left atria were transected to allow escape of the perfusate which was collected by suction from the thoracic cavity. The pulmonary vasculature was also perfused via the right ventricle and pulmonary artery. A similar procedure using 1·5% cacodylate buffered Glutaraldehyde was used for tissue fixation in some experiments.

Tissues were then obtained by dissection, weighed and processed for estimation of total radioactivity by liquid scintillation counting for β-ray emitters in a Beckman LS 250

* PBS, Dulbecco's phosphate buffered saline.

TABLE 2. Comparison of isotopes. Tissue radioactivity 24 hr after i.v. injection of labelled TDLs into syngeneic recipients (perfused)

Tissue	Weight or volume factor (g or ml)[e]	Per cent of injected dose per organ								
		^{3}H Udr (8)[d]	^{14}C Udr (4)	^{3}H Leu (1)	^{14}C Leu (1)	^{75}Se L-Sem (6)	^{99m}Tc Na_2TcO_4 (24)	^{51}Cr Na_2CrO_4 (50)	^{3}H Tdr (*in vivo*) (6)	^{125}I Iudr (10)
Lymphoid tissues										
Spleen	0·4 g	7·53	11·1	4·92	6·78	6·29	8·12	18·0	14·1	2·71
Superficial lymph nodes[a]	0·5	8·81	9·89	7·96	7·96	11·4	3·99	30·7	30·6	1·93
Small intestine[b]	5·0	2·99	2·74	7·04	1·94	5·31	n.d.	2·42	3·17	25·0
Thymus	0·3	0·08	0·11	0·19	0·09	0·12	0·009	0·07	0·05	0·08
Bone (marrow)[c]	10·0	n.d.	n.d.	n.d.	n.d.	1·95	n.d.	5·17	n.d.	4·30
Non-lymphoid tissue										
Liver	10·0	3·9	5·7	21·6	5·6	7·0	18·9	11·4	2·1	3·4
Kidney	1·5	0·59	0·46	0·72	0·44	1·48	6·69	0·58	0·26	0·18
Lung[f]	1·2	1·41	1·70	1·47	2·22	2·01	0·34	2·81	4·02	1·31
Skin	30·0	2·79	1·31	5·37	4·29	3·69	2·64	0·38	2·48	2·31
Muscle	100·0	13·2	5·33	17·1	7·50	9·80	2·60	0·85	10·9	2·16
Ovary	0·1	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	0·009	n.d.	0·018
Uterus	0·4	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	0·008	n.d.	0·064
Testis	2·5	0·54	0·09	0·60	0·27	0·69	n.d.	0·026	0·20	0·092
Epididymis	3·5	0·22	0·11	0·54	0·18	0·28	n.d.	0·051	0·17	0·20
Salivary gland	0·3	0·08	0·08	0·11	0·04	0·09	n.d.	0·012	0·04	0·04
Brain	1·5	0·16	0·04	0·22	0·21	0·18	n.d.	0·004	0·05	0·018

Fluids										
Whole blood	15·0 ml	4·33	1·76	5·55	3·93	9·44	5·51	3·62	14·3	1·76
Plasma	9·0	4·32	0·39	5·94	1·98	8·15	0·76	0·33	2·69	1·09
White blood cells[g]	15·0	n.d.	1·83	n.d.	n.d.	n.d.	n.d.	1·88	n.d.	0·28
Whole perfusate[h]	250	16·2	2·72	n.d.	n.d.	n.d.	n.d.	1·18	n.d.	3·18
Supernatant perfusate[i]	250	25·6	1·45	35·0	2·66	3·11	n.d.	0·35	9·38	2·68
Cells from perfusate[j]	250	2·54	1·20	0·53	0·67	0·92	n.d.	1·28	n.d.	0·25
Approx. total recovery		66	40	95	41	59	46	73	79	47

[a] Lymph nodes other than the mesenteric nodes.
[b] Proximal jejunum avoiding Peyer's Patches.
[c] Tibiae and femora counted whole.
[d] Numbers in parentheses indicate the number of recipients.
[e] Total weight or total volume of organ in 'standardized' 200 g rat.
[f] Peripheral lung parenchyma avoiding major bronchi.
[g] Separated on Ficoll/Hypaque gradient.
[h] Unseparated.
[i] Cell free supernatant after centrifugation of (h).
[j] Cell pellet of (i).

Spectrometer or by direct counting in a well type γ-scintillation counter (L.K.B. Wallac 1280 Ultragamma) for γ-ray emitters.

Heparinized blood samples were divided; 2·0 ml were diluted with 8·0 ml PBS and layered on 3·0 ml of Ficoll (67 ml, 14%)/Hypaque (20 ml, 45%) mixture, centrifuged at 1200 *g* for 30 min at 15°C and the interface layer was collected for counting of blood white cell activity. A further 2·0 ml was centrifuged at 1500 *g* for 10 min to obtain plasma.

Samples were processed for liquid scintillation counting by the method of Ford & Hunt (1973) with one modification. The addition of hydrogen peroxide, necessary to bleach pigment and therefore reduce colour quenching in many tissues, was found to cause spurious counts in the low energy (^{3}H) range when clear fluids, e.g. serum were examined, presumably due to incomplete release of oxygen. This was most efficiently counteracted by the addition of 0·2% rat blood to the sodium hydroxide digestion mixture. The maximum weight of tissue sample that can be emulsified in 15 ml of scintillation cocktail with this method is 50 mg and this may prevent counting of tissues containing very few labelled cells. Much larger samples (1 g, approximately) of such tissues can be counted directly in a gamma counter. β and γ count rates of less than 2 SD's above mean background values were excluded and β count rates above background were converted to d.p.m.

Autoradiography was performed on routine histological sections using Ilford G5 dipping emulsion.

RESULTS

Comparison of radioisotopic labels

The object of these experiments was to find the most suitable isotope for tracing lymphocytes into non-lymphoid tissues allowing for the unsuitability of histological autoradiography in this context. Examination of many sections of tissues of recipients of large numbers (up to 2×10^9) of ^{3}H or ^{14}C labelled TDLs confirmed that labelled cells could be found in the interstices of all tissues examined but at too low a frequency to allow any meaningful attempt at quantitation. Table 2 shows the distribution of radioactivity 24 hr after i.v. injection of syngeneic TDLs labelled with the radioisotopes which are commonly used for this purpose. In each case tissues were sampled after whole body perfusion (see Methods). The data were pooled from several experiments and expressed as the arithmetic mean percentage injected activity per whole tissue. The total weight of each tissue was derived by dissection of several animals and from these measured weights the approximate weight of each tissue in a 'standard' 200 g rat was computed and is stated in Table 2.

Within an experiment using the same cell suspension, the standard deviation about the mean of activities in quadruplicate recipients was between 10 and 30% of the mean. However, when the results of several experiments were pooled the variation increased to give a standard deviation of approximately 50% of the mean. The SD values have been excluded from the tables for the sake of clarity. There were large differences in the measured activity in each tissue when estimated with different labels and the important comparisons are considered separately.

A. Tritium vs carbon 14. The pattern of distribution following injection of lymphocytes incubated with the same molecule labelled with different radioactive atoms e.g. [^{3}H] or [^{14}C] uridine or leucine was markedly different. ^{3}H gave higher levels of activity in non-lymphoid

TABLE 3. Ratio of percentage of injected ^{3}H/injected ^{14}C in tissues 24 hr after injection of thoracic duct lymphocytes double-labelled with ^{3}H and ^{14}C uridine

Tissue	Recip. A	Recip. B
Injected cells	1·0	1·0
Spleen	0·82	0·80
Lymph node	0·93	0·88
Small intestine	0·89	0·86
Thymus	0·73	0·73
Liver	0·94	0·95
Lung	0·85	0·82
Kidney	1·20	1·29
Testis	4·31	5·89
Epididymis	1·81	2·90
Muscle	2·32	2·17
Brain	4·61	8·19
Skin	1·62	2·06
Plasma	4·7	4·7
Supernatant perfusate	18·6	19·3

tissues such as muscle when compared with ^{14}C but the reverse was true in spleen and lymph nodes, areas where many labelled lymphocytes might be expected. These observations were confirmed in an experiment using double-labelled cells where the results were expressed as the ratio of $^{3}H/^{14}C$ in each tissue (Table 3).

To test the possibility that the two isotopes were eluting from cells at different rates, double-labelled cells were injected intravenously into a recipient with an indwelling thoracic duct cannula. The radioactivity associated with the cells and the cell-free supernatant lymph in successive 90 min collections of draining lymph was measured. Fig. 1a,b shows the results as the percentage of injected dose for each isotope for each collection period.

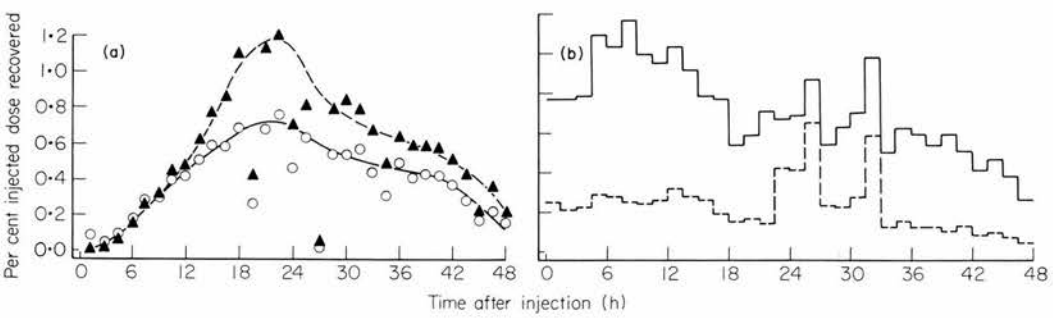

FIG. 1. (a) Lymphocyte associated radioactivity (as per cent of injected activity) in sequential 90 min collections of lymph from a rat with a thoracic duct fistula which received at time zero a 'bolus' i.v. injection of syngeneic thoracic duct lymphocytes, double-labelled *in vitro* with ^{3}H (○) and ^{14}C (▲) uridine. (b) In the same experiment as (a) the supernatant cell free lymph radioactivity was also measured in sequential 90 min collections and the results are expressed as per cent injected activity for each isotope for each collection period. Bold line, ^{3}H; dotted line, ^{14}C.

The pattern of cell-associated activity was very similar for both isotopes but the absolute quantity of ^{3}H recovered over 48 hr was significantly less than ^{14}C, in agreement with Ford & Simmonds (1972). However, the cell-free supernatant lymph contained substantial quantities of tritium very soon after injection and before any activity was associated with the cell pellet and these levels showed a gradual decline with time. These observations of high levels in non-lymphoid tissues, plasma and particularly the cell free supernatant of the perfusate suggested widespread redistribution of eluted tritium. The total perfusate ^{3}H activity was considerably higher than the maximum estimates of total blood activity at 24 hr suggesting removal of activity from the extravascular compartment. This was also shown by the significant reduction in tissue activity by perfusion which was not seen with other labels, e.g. ^{51}Cr.

B. [^{51}Cr] *sodium chromate.* The radioactivity measured in lymph nodes due to ^{51}Cr was as high as with [^{3}H]thymidine (*in vivo*) while the levels in all non-lymphoid tissues were consistently the lowest of the isotopes used. Very little activity was measured in plasma or cell-free perfusate suggesting that any eluted label is efficiently removed and probably not reutilized. In support of this was the observation that when ^{51}Cr-labelled cells were heat-killed prior to i.v. injection, the majority of label was recovered in the liver and the proportion so localized remained constant from 2–48 hr while the remaining activity was mainly in plasma and this level fell steadily with time, this fall being associated with urinary excretion.

The considerable activity in liver after injection of viable cells also remained constant with time and thus it is impossible to be sure how much of this activity was due to physiological localization of normal cells and how much may be a reflection of a proportion of damaged cells in the original population following labelling.

C. [^{75}Se]-L-*selenomethionine.* This substituted amino acid is incorporated by lymphoid cells without impairing their functional capacity (Bainbridge, 1976; Rose & Micklem, 1976). However, as might be expected, the isotope eluted from cells and several findings indicate that the label was reutilized. (i) High plasma activity which was found to be T.C.A. insoluble. (ii) High non-lymphoid tissue concentrations particularly in the small intestine (cfd with ^{51}Cr). (iii) The redistributed label was not removed by perfusion, as the activity in the perfusate could be accounted for by the blood alone.

D. [^{99m}Tc] *sodium pertechnetate.* Sodium pertechnetate as a lymphocyte label has been reported to have similar characteristics to sodium chromate although such potentially toxic compounds as stannous chloride were required in the *in vitro* incubation procedure to maintain stability of labelling (Barth, Singla & Gillespie, 1974). In the present studies the addition of stannous chloride or sodium chromate resulted in only a marginal increase in label uptake. The behaviour of such labelled cells *in vivo* was inferior to those labelled with ^{99m}Tc alone.

Tissue activity showed great variation between experiments. Low levels were noted in lymph nodes and the ability of labelled cells to migrate from blood to thoracic duct lymph was markedly impaired. High concentrations of label were found in liver and kidney. These results all suggest a significant degree of cell damage and label elution which was also reflected by massive urinary excretion.

E. [^{3}H] *thymidine.* Labelled long-lived lymphocytes obtained by repeated administration of [^{3}H]thymidine to prospective TDL donors should have a stable DNA-associated label and the trauma of *in vitro* manipulation for transfer experiments is minimized by such a labelling procedure.

High concentrations were indeed measured in lymph nodes but elution problems similar to those associated with [^{3}H]uridine and [^{3}H]leucine were encountered as shown by the

TABLE 5. The kinetics of tissue localization of radioactivity following intravenous injections of ^{51}Cr or ^{125}I labelled TDL into syngeneic recipients (perfused)

Tissues	Weight* or volume† factor	Percentage of injected dose per organ								
		Normal TDL ^{51}Cr			Passaged TDL ^{51}Cr			Normal TDL ^{125}I		
		(0·5)‡	(2)	(24)	(0·5)	(2)	(24)	(0·5)	(2)	(24)
Lymphoid tissues and fluids										
Spleen	0·4 g	34·1	42·1	18·0	46·8	56·8	17·1	11·0	9·9	2·7
Superficial lymph nodes	0·5 g	4·1	4·7	30·7	6·5	7·7	25·9	0·88	0·75	1·93
Mesenteric lymph nodes	0·15 g	0·89	1·52	6·55	1·35	2·04	6·35	0·39	0·76	0·66
Small intestine	5·0 g	1·77	2·40	2·42	0·93	0·56	1·54	13·7	18·9	24·9
Thymus	0·3 g	0·013	0·014	0·073	0·006	0·008	0·026	0·067	0·063	0·082
Bone (marrow)	10·0 g	24·9	25·7	5·2	7·9	16·9	2·4	18·8	16·4	4·3
White blood cells	15·0 ml	3·26	0·70	1·88	15·1	3·1	1·7	2·1	0·48	0·28
Whole blood	15·0 ml	4·75	1·53	3·62	13·9	2·9	2·7	6·57	2·19	1·76
Plasma	9·0 ml	0·73	0·50	0·33	0·54	0·16	0·19	2·33	1·32	1·09
Supernatant perfusate	250 ml	0·75	0·40	0·35	1·02	0·10	0·12	6·88	4·65	2·67
Cells in perfusate	250 ml	4·22	0·50	1·28	5·63	1·48	0·73	2·15	0·80	0·25
Non-lymphoid tissues										
Lung	1·2 g	17·3	3·6	2·8	24·2	3·1	2·2	34·9	6·9	1·3
Liver	10·0 g	12·3	11·4	11·4	15·8	8·9	9·3	28·4	15·9	3·4
Kidney	1·5 g	0·49	0·45	0·58	0·68	0·34	0·52	0·64	0·22	0·18
Ovary	0·1 g	0·023	0·011	0·009	0·020	0·003	0·003	0·052	0·021	0·018
Uterus	0·4 g	0·004	0·009	0·008	0·007	0·002	0·004	0·179	0·075	0·064
Testis	2·5 g	0·013	0·015	0·025	0·049	n.d.	n.d.	0·133	0·111	0·092
Epididymis	3·5 g	0·049	0·040	0·051	0·179	n.d.	n.d.	0·330	0·105	0·200
Muscle	100·0 g	1·78	1·13	0·85	3·21	1·11	0·38	7·21	3·22	2·16
Salivary gland	0·3 g	0·004	0·004	0·012	0·030	0·003	0·017	0·036	0·015	0·039
Brain	1·5 g	0·009	0·006	0·004	0·022	0·009	0·002	0·025	0·010	0·018
Skin	30·0 g	0·99	0·54	0·39	1·11	0·33	0·18	5·49	2·49	2·31

* Total weight of organ in 'standardized' 200 g rat.

† Total volume for 'standardized' 200 g rat.

‡ Figures in parentheses indicate time in hours.

following observations. (i) Levels in non-lymphoid tissue were about an order of magnitude greater than those measured with ^{51}Cr. (ii) Plasma and supernatant perfusate levels were high with the latter being in excess of the total plasma activity.

F. [^{125}I]iododeoxyuridine. In vitro incubation with this analogue of thymidine was used to label DNA-synthesizing lymphoblasts in normal thoracic duct lymph. Such cells comprise up to 5% of the whole population and are generated for the most part in the area drained by the thoracic duct, i.e. mainly mesenteric lymph node and gut associated lymphoid tissue.

Following i.v. injection of ^{125}I-Udr labelled cells there was a steady decrease in total recoverable label associated with urinary excretion. High plasma levels and slightly more label in the cell free perfusate than in the total blood volume suggest that a proportion of the eluted label is widely distributed. Absolute levels of ^{125}I activity in non-lymphoid tissues may therefore overestimate the cell content. However, after taking into account the effect of this contamination, there still appeared to be a greater proportional distribution of lymphoblasts compared to ^{51}Cr-labelled whole TDLs in non-lymphoid sites.

The changes in tissue distribution of labelled cells with time

The migration of populations of lymphocytes was studied by sampling tissues at various times after i.v. injection of a 'bolus' of labelled cells. Tissues removed at $\frac{1}{2}$ and 2 hr after

TABLE 4. Late time course of tissue radioactivity following i.v. injection of normal ^{51}Cr TDL into syngeneic recipients (perfused)

Tissue	Percentage of injected dose per gram of tissue		
	24 hr	48 hr	72 hr
Spleen	42·5	26·5	28·9
Superficial lymph nodes	65·5	53·9	49·6
Mesenteric lymph node	52·9	55·3	41·9
Small intestine	0·65	0·70	0·51
Thymus	0·37	0·26	0·30
Bone (marrow)	0·78	0·53	0·49
Liver	1·4	1·2	1·4
Kidney	0·33	0·33	0·38
Muscle	0·004	0·006	0·007
Skin	0·011	0·010	0·010
Brain	0·003	0·0008	0·0004
White blood cells	0·153	0·070	0·086

Values are means of three recipients of the same cell suspension at each time point.

injection reflect the distribution associated with the initial emigration of cells from the blood while 24 hr samples reflect the distribution at near-equilibration within the 'recirculating lymphocyte pool', as suggested by the small fluctuation of any tissue concentrations noted after this time (Table 4).

Three populations were studied using ^{51}Cr and ^{125}I as these isotopes were shown to have fewer disadvantages for this type of study particularly in non-lymphoid sites: (1) whole

TDLs–^{51}Cr-labelled cells comprising lymphocytes of all types; (2) passaged TDLs–^{51}Cr-labelled cells depleted in labelled lymphoblasts (see Methods); (3) lymphoblasts–^{125}I-Udr labelled *in vitro*.

Table 5 shows the pooled results of several experiments using these three populations expressed as the mean percentage injected dose per whole tissue after whole body perfusion.

(i) The spleen accounted for the majority of the injected label (less so for ^{125}I) for the first 2 hr and the concentration then fell with equilibration by approximately 48 hr (Table 5).

(ii) The lymph nodes showed an early steep rise (approx. 8%/hr for the first half hour) followed by a slower but progressive accumulation (average 1%/hr between two 24 hr) with maximal levels at approximately 24 hr. The injection of ^{125}I-labelled lymphoblasts was followed by a three times greater concentration of label (per unit weight) in the mesenteric lymph nodes compared with superficial lymph nodes (i.e. nodes draining sites other than the intestine) at 2 hr but not later.

(iii) The bone marrow accounted for a considerable proportion of the injected label with a temporal pattern similar to that in spleen.

(iv) The small intestine (excluding Peyer's Patches) accumulated a large proportion of ^{125}I-labelled lymphoblasts with a steady increase in concentration. Passaged ^{51}Cr-labelled cells also showed significant levels of activity in the small gut with slight accumulation by 24 hr.

(v) In the thymus radioactivity accumulated with a pattern similar to lymph nodes but at much lower concentrations. These concentrations were significant and on a per gram basis were higher than those of all non-lymphoid tissues except liver and kidney by 24 hr. The presence of labelled cells was confirmed by autoradiography of ^{3}H-labelled TDLs which showed such cells located in the thymic medulla.

(vi) In blood the majority of the activity following injection of ^{51}Cr-labelled whole TDLs was associated with cells. The maximum level was soon after injection and this was followed by a rapid fall in concentration with a minimum at 2–3 hr and a slight rise at 24 hr. However, with ^{125}I-labelled cells the label measured in blood was mainly in the plasma and the cell-associated activity fell rapidly and progressively after injection.

(vii) Non-lymphoid tissues had low concentrations at all times with maximum activity soon after cell injection and rapid equilibration in a similar manner to blood. Minimum concentrations were sometimes measured in, e.g. skin and muscle at 2–3 hr, and this was particularly noticeable within individual experiments using the same cell suspension. Central nervous tissue consistently gave the lowest concentrations of activity, which might be expected of a tissue lacking a lymphatic drainage.

In an attempt to accentuate these possible fluctuations in non-lymphoid tissue activity by maintaining higher blood concentrations, experiments were performed using splenectomized recipients; the rationale being that as approximately half the injected cells are at first removed from the circulation by the spleen in the intact animal then removal of this 'sink' would allow more cells a chance to migrate to other sites.

Table 6 shows the effect of splenectomy on the distribution with time of ^{51}Cr label associated with i.v. injected TDLs expressed as the percentage of injected activity per gram of tissue. The rate of fall of blood leucocyte activity was decreased compared with that in intact animals. Most tissues showed higher concentrations of label, particularly soon after injection, but this effect was minimal in non-lymphoid tissues. The reduced rate of decline of the early high levels in the lung was marked and the significance of this observation will be discussed. These results also accentuated the capacity of the bone marrow to accept large numbers of lymphocytes from the circulation.

TABLE 6. Effect of splenectomy on recipients of ^{51}Cr labelled syngeneic normal TDLs

	Percentage of injected dose per gram of tissue					
	0·5 hr		2 hr		24 hr	
Tissue	Intact	Splenect.	Intact	Splenect.	Intact	Splenect.
Lung	14·4	40·4	3·01	9·37	2·34	2·75
White blood cells*	0·217	0·854	0·047	0·145	0·125	0·115
Spleen	85·1	—	105·0	—	45·1	—
Superficial lymph nodes	8·13	33·5	9·45	30·1	61·4	80·0
Mesenteric lymph node	5·92	10·1	10·1	22·8	43·7	63·4
Bone (marrow)	2·50	4·78	2·57	7·09	0·52	1·47
Thymus	0·044	0·084	0·047	0·076	0·245	0·375
Small intestine	0·355	0·890	0·479	0·752	0·484	1·26
Liver	1·23	2·35	1·14	2·02	1·14	1·62
Kidney	0·323	1·33	0·297	0·402	0·388	0·425
Muscle	0·018	0·031	0·011	0·014	0·009	0·015
Brain	0·017	0·018	0·009	0·014	0·008	0·004
Skin	0·033	0·041	0·018	0·026	0·013	0·012

* Per ml of blood separated on Ficoll/Hypaque.

DISCUSSION

1. Lymphocyte markers

Any method of estimating lymphocyte migration *in vivo* requires lymphocytes to be 'marked' so that they may be recognized in other tissues. Such markers should be permanent, non-injurious and have such activity that detection of small numbers is possible. No such ideal label exists. Chromosome markers are permanent and non-toxic but have the great disadvantage of being visible only in dividing cells and requiring laborious microscopic scoring. Radioisotopes provide a convenient and objective measure of tissue radioactivity after labelled cell injection but equation of such tissue activity with the presence of cells requires that the isotope remains attached to the injected cells, and that these remain alive. In practice this is never completely realized and therefore the usefulness of any isotope as a cell marker is related directly to the degree of toxicity of the labelling method, the extent of label elution from dead and living cells and the subsequent fate of such eluted label. These factors are particularly important where the expected concentration of labelled cells is low, i.e. they may limit the resolution of the system.

The data presented underline the limitations of several radioactive lymphocyte labels when used to quantify *in vivo* migration.

(i) Tritiated compounds have the severe disadvantages of large scale elution and more significantly of widespread redistribution and persistence in tissue of label which yield gross overestimates of cell concentrations in non-lymphoid tissues, making such labels entirely unsuitable for quantitative studies in such sites.

The increased elution of tritium compared with ^{14}carbon from lymphocytes double-labelled with [^{3}H] and [^{14}C]uridine or leucine reflects the different metabolic fates of atoms within a molecule. This can be explained by different pathways of catabolism with intracellular conservation of ^{14}carbon, or possibly by exchange of tritium for hydrogen at the surface of the molecule.

The eluted tritium appears to move within the body water pool. In restrained rats receiving a continuous intravenous infusion of normal saline approximately 30% (Fig. 1b) to 50% (Goldschneider & McGregor, 1968) of the injected activity was found in cell free lymph or urine over 24–48 hr after [^{3}H]uridine-labelled cell injection. However, in similarly restrained rats with no infusion and therefore no excess water input we found only 3% of the injected activity was recovered in urine over 24 hr after similar labelled cell injection.

This widespread redistribution of TCA-soluble tritiated material could provide an alternative explanation of the fact that ratios of TCA-insoluble/TCA-soluble tritium increase with time in lymphoid tissues but not elsewhere (Goldschneider & McGregor, 1968; Ellis & de Sousa, 1974). Such results have been cited as evidence that lymphocytes continue to synthesize radioactive RNA from TCA-soluble precursors after injection of *in vitro* labelled cells only in lymphoid tissue. However, such alterations in this ratio would also arise if the TCA-insoluble fraction increased due to accumulation of more labelled cells with time while the TCA-soluble fraction decreased by elution and redistribution. Both these factors are in fact operative.

The situation differs in non-lymphoid tissues because the influx of cell free TCA-soluble tritium from net elution in lymphoid organs may more than balance the efflux of TCA-soluble tritium from the few labelled cells in the tissue, resulting in no increase or even a decrease in the TCA-insoluble/TCA-soluble ratio.

The autoradiographic evidence of an increase in TCA resistant grain count over TDLs more than 27 hr after injection compared with the same population immediately after labelling (Gowans & Knight, 1964) suggests that a continuation of labelled RNA synthesis can occur but there is no convincing evidence of restriction of this ability to cells in lymphoid tissue.

(ii) ^{14}Carbon has advantages over tritium due to the lower contamination of tissues by eluted label which may be due in part to excretion of catabolized label as $^{14}CO_2$.

(iii) [^{51}Cr]sodium chromate. Of the markers investigated ^{51}Cr has most advantages and fewest disadvantages in the present context. The disadvantages are the following. (a) Toxicity. The ability of ^{51}Cr-labelled mouse lymphoid cells to survive and respond to mitogenic stimulation *in vitro* after transfer to syngeneic recipients is reduced compared with similar unlabelled cells as measured by chromosomal markers (Doenhoff & Davies, 1976). This toxicity may explain in part the higher liver activity found with this label. Low labelling concentrations must be used to minimize this effect. (b) Non-specific label uptake. This is only a disadvantage if the starting population of cells is heterogeneous. Preliminary studies provided evidence that up to 10% contamination with erythrocytes could be found in thoracic duct lymph in the absence of obvious bleeding. Such contamination could be effectively removed by centrifugation on a Ficoll/Hypaque gradient, with good recovery of lymphocytes and no loss of viability. Ammonium chloride lysis proved a much less efficient method of removing small numbers of erythrocytes. (c) Autoradiography. Good autoradiographs of ^{51}Cr-labelled cells were obtained with cell smears but tissue sections gave much poorer results with apparent loss of activity during processing. This limits studies of the precise anatomical location of ^{51}Cr-labelled cells. (d) Non-uniformity of label uptake. Large lymphocytes are

labelled more heavily than small lymphocytes by approximately 2:1 (Eyre, Rosen & Perry, 1970). Thoracic duct lymph contains about 5% large lymphocytes and it is therefore impossible to estimate accurately how much of a given tissue activity is due to this sub-population of cells if they behave differently as the evidence suggests.

On the other hand the advantages of easy and efficient counting of the emitted γ-rays and good elution characteristics with low contamination of tissues by non-cell associated label make this label very useful. ^{51}Cr gives estimates of activity in non-lymphoid tissue that are most likely to approximate to physiological levels. In fact the cell concentrations in tissues may be underestimated with this label following net elution and excretion of free label but is unlikely to be overestimated as is the case with the other labels studied.

(iv) The potential advantage of [^{75}Se]-L-selenomethionine as an alternative γ-emitting isotope that has been shown not to impair lymphocyte function (Rose & Micklem, 1976) is counteracted by the rapid and possibly uneven elution from different organs (Bainbridge, 1976) together with some degree of redistribution probably due mainly to reutilization. Thus ratios between ^{51}Cr and ^{75}Se vary between lymphoid tissues with time unlike the ratio of ^{3}H/^{14}C in similar double-labelling studies.

(v) [^{3}H]thymidine. Although thymidine is metabolically incorporated into DNA and therefore should be stable throughout the life of the cell significant elution of tritium does occur from transferred long-lived cells. This can be explained by the death of a small proportion of the labelled cells, catabolism of a proportion of so called 'metabolic DNA' (Harris, Pelc & Blackmore, 1973) or a more uniform elution of isotope by atomic exchange as suggested for other tritiated compounds.

The expense in terms of isotope and time needed to label long-lived cells *in vivo* together with the contaminatory elution of isotope in final recipients of labelled cells override the potential advantage of transferring a less traumatized population, for quantitative studies in non-lymphoid tissues.

(vi) [^{99m}Tc]sodium pertechnetate. With the *in vitro* labelling methods used (after Barth *et al.*, 1974) the potential advantages of the short half life (6 hr) and good imaging characteristics of the γ-emission of ^{99m}Tc were nullified by the apparent toxicity as shown by poor cell survival and function *in vivo*.

(viii) [^{125}I]iodo-deoxyuridine. As a label for DNA synthesizing cells this analogue of thymidine has the advantage over, e.g. [^{3}H]thymidine of being a γ-emitter. It also has slightly better elution characteristics but there is a significant degree of tissue contamination and this isotope is also inherently toxic to cells, as shown by the four to five times greater radiation damage by ^{125}I-Udr compared with ^{3}H-Tdr (Hofer & Hughes, 1971) and also the changing migration characteristics of murine 'activated T.TDL' labelled with increasing doses of ^{125}I-Udr, with poorer splenic localization reflecting probable cell damage (Sprent, 1976). Thus 1 μCi/ml is the upper limit of an acceptable *in vitro* labelling dose.

2. Kinetic studies

The behaviour of inherently mobile cell populations is revealed by the changing patterns of distribution of such cells with time after i.v. injection. After equilibrium has been achieved the relative concentrations of activity in the tissues provide evidence of the proportional size of the various compartments of the recirculating lymphocyte pool (RLP) if the following assumptions are true. (a) The cells injected are part of and mix freely within the total pool *in vivo*. (b) The cells behave in a uniform manner. This is not true for mixtures of large and small

lymphocytes nor for mixtures of T and B lymphocytes (Nieuwenhuis & Ford, 1976; Freitas & de Sousa, 1976). (c) Distortion due to loss of label and cell death are not too great.

The early changes in concentration of activity in tissues with time following a bolus injection of marked cells yield clues about the proportional distribution of lymphocytes entering the blood (e.g. from major lymphatic vessels), i.e. the likelihood of a given cell finding itself able to extravasate at a given site. The latter is thus a function of blood concentration, blood flow and a clearance factor which varies between tissues and may be a property of venular endothelium.

The time taken to reach maximum levels of radioactivity always assuming this is cell associated and the rate of decrease from this maximum must reflect the modal transit time of lymphocytes through a tissue.

A. ^{51}Cr TDLs. The kinetic pattern of distribution of passaged ^{51}Cr TDLs is generally similar to that of ^{51}Cr whole TDLs and these two populations will therefore be considered together.

(1) Blood and lungs. The labelled cells are injected into the blood compartment and thus maximal blood levels are achieved immediately. The rate of fall is rapid and a minimum is attained at 2–3 hr with a rebound by 24 hr which is the same pattern as described by Bunting, Kiely & Owen, 1963. This initial fall is associated with a high concentration in the lungs and this has been explained by micro-embolization or retention of cells, traumatized by *in vitro* manipulation, in the first capillary bed reached (Gowans & Knight, 1964; Hall, Scollay & Smith, 1976).

However, such apparent hold-up in the lungs may reflect a physiological process rather than an artefact. This is suggested by two observations. Intra-arterial injection of labelled cells, which therefore must traverse a capillary bed before reaching the lungs, yields an early distribution pattern including high lung levels essentially similar to that following i.v. injection (Weisberger *et al.*, 1951; Rannie, unpublished). Secondly the initial rapid fall in lung levels is significantly slowed in splenectomized recipients whether the operation is performed immediately or 10 days before labelled cell transfer. Splenectomy removes a tissue which accepts large numbers of cells from the circulation and following removal of this 'sink' the blood concentration remains higher for longer after i.v. injection of labelled cells. That the lung levels are also maintained under such circumstances can only be explained if the cells in the lung are part of the mobile circulatory pool and not temporarily sequestered. If lymphocytes traverse the lung capillary bed slowly (i.e. measured in minutes rather than seconds) the observed activity levels would be expected as many labelled cells would return to the lungs of necessity having failed to extravasate elsewhere.

(2) Secondary lymphoid organs. The kinetics of migration in lymph nodes and spleen confirm the observations of others in intact and splenectomized recipients (Gowans & Knight, 1964; Goldschneider & McGregor, 1968; Ford, 1969; Bradfield & Born, 1973). These tissues therefore acted as a positive control suggesting normal behaviour of the injected cells.

(3) Primary lymphoid organs. (a) Thymus. Localization of significant quantities of radioactivity following injection of labelled large or small lymphocytes shows that the barrier to recirculating cells in this tissue is not complete. On a per gram basis at equilibrium the thymus shows concentrations intermediate between lymph nodes and most non-lymphoid tissues. Autoradiographs using [^{3}H]uridine-labelled cells showed these labelled cells to be mainly in the thymic medulla and such cells were most easily visualized after large doses of labelled

lymphocytes although the proportion of the injected dose remained constant. This may explain the results of Gowans & Knight (1964) who found labelled small lymphocytes in the thymuses of young rats but not of adults. Werdelin (1972) reported localization of newly formed cells from antigen stimulated lymph nodes labelled *in situ* with [^{3}H]thymidine in the thymic medulla. The observation of accumulation of passaged ^{51}Cr-labelled cells as well as ^{125}I-labelled blasts suggests that accredited recirculating cells also have this capability.

(b) Bone marrow. In the normal rat a small proportion of bone marrow cells share the characteristics of recirculating small lymphocytes, such as graft versus host activity of bone marrow (Yoshida & Osmond, 1971) which can be removed by prolonged thoracic duct drainage (Howard & Scott, 1972). As early as 1951, Farr showed substantial localization of fluorescent-labelled lymph node cells in rabbit bone marrow a few hours after intravenous injection which was no longer apparent at 12 hr. This subsequent disappearance was misinterpreted as being due to cell death or transformation as opposed to remigration. Using ^{51}Cr-labelled lymphocytes Shorter & Bollman (1960) give a maximum bone marrow localization of 4% of injected dose but do not give the size of the sample. The present data suggest that a major pathway of recirculating lymphocytes is through the bone marrow with a transit time similar to that in spleen, i.e. 4–6 hr (Ford, 1969). The present estimates of 25% of the injected cells in the bone marrow at 2 hr is computed assuming that 5% of body weight comprises bones containing marrow to the same extent as the measured tibiae and femora. That labelled cells enter and leave the marrow parenchyma in all sites was confirmed by autoradiography of decalcified histological sections of perfused recipients at several times after transfusion of ^{3}H-labelled TDLs.

Recognition of this major recirculation pathway has been masked by the diffuse nature of the tissue, and the large extent of lymphocyte production that occurs, rendering long-lived recirculating cells a small minority in the equilibrium state. It remains to be shown that this recirculation of lymphocytes between the blood and the bone-marrow has similar functional significance to recirculation through the spleen and lymph nodes in the propagation of immune responses (Gowans & McGregor, 1965, reviewed by Ford, 1975).

(4) Non-lymphoid tissues. In non-lymphoid tissues, the early low peak, the slight fall after a few hours and rapid equilibration all follow the blood curve. This suggests a low clearance factor and a rapid transit time of the cells that do extravasate. An alternative explanation of the close association of blood and tissue concentrations could be that the latter is due to intravascular-labelled cells incompletely removed by perfusion. If this is the case the tissue levels measured would overestimate cell extravasation. Against this are the observations that: (i) some vascular tissues, e.g. brain, can be perfused to give much lower levels than, e.g. skin, even at early time intervals, when the blood concentration is high. (ii) At 2 hr although ^{51}Cr and ^{125}I concentrations in most tissues are lower than at $\frac{1}{2}$ hr, this decrease is much less dramatic than that measured in blood leucocytes over the same period.

The problem of transit time in peripheral tissues can only be approached directly by collecting labelled cells as they leave such tissues via afferent lymphatic fistulae. In sheep it has been shown that some labelled lymphocytes do emerge from afferent lymphatics soon after i.v. injection (Hall *et al.*, 1976). Presentation of the data as specific activity in afferent and efferent lymph makes estimation of the true kinetics difficult without cell counts or reference to a fixed standard such as the injected dose. However, such curves showed that the afferent lymph curve anticipates that of efferent lymph by approximately 6 hr, and also that significant activity was present in afferent lymph within the first 2 hr.

Using the shape of the time course distribution curves to estimate transit time and concentration at equilibrium to estimate the proportion of the RLP in a tissue an indirect estimate of cell flux through that tissue may be made. For example, from the present data, using passaged ^{51}Cr-labelled cells as an accredited recirculating population, one gram of skin in a 200 g rat contains approximately 0·006% of the RLP. Assuming the latter to be in the region of $2{\cdot}5 \times 10^9$ cells (defined from the total cells mobilizable into the thoracic duct over 4 days by which time the output curve has reached plateau levels (after Gowans & Knight, 1964)) then approximately $1{\cdot}5 \times 10^5$ recirculating lymphocytes are present in 1 g of skin at any given time. Depending on the mean transit time of this population, a proportion of the cells will enter and leave the tissue each hour. If the transit time is, e.g. 3 hr, as suggested by the rapid equilibration shown in this report, then one third of the population will enter each hour or 5×10^4 cells/g/hr. Whereas if the transit time was 10 hr (Hall *et al.*, 1976) then this flux would be reduced to $1{\cdot}5 \times 10^4$/g/hr.

This size of flux agrees reasonably closely with estimates computed from afferent lymphatic cell output data in sheep (Smith, Pederson & Morris, 1970). Renal afferent lymph contained approximately $2{\cdot}0 \times 10^6$ lymphocytes/hr which, assuming the weight of the kidney to be 100 g, would yield a figure of $2{\cdot}0 \times 10^4$/g/hr. The testis had an output of $5{\cdot}0 \times 10^6$/hr which, assuming a weight of approximately 250 g, gives a flux value of $2{\cdot}0 \times 10^4$/g/hr. The liver had a higher concentration of cells in afferent lymph with a total output of 75×10^6 lymphocytes/hr. If the total weight of tissue was approximately 1500 g the flux would be 5×10^4/g/hr. A reasonable estimate of tissue flux would therefore be 10^4–10^5 lymphocytes/g/hr.

B. ^{125}I-labelled lymphoblasts. The pattern of distribution of labelled lymphoblasts corresponds closely with that reported by others (Hall, Parry & Smith, 1972; Gowans & Knight, 1964) and suggests that large cells have a greater propensity for leaving the circulation at all non-lymphoid sites. However, on a cell-for-cell basis, comparison of this population with passaged ^{51}Cr cells which contain few labelled lymphoblasts suggests that the absolute numbers of large and small lymphocytes extravasating in non-lymphoid tissues including gut are roughly equal.

The approximately three times higher concentration of ^{125}I in the mesenteric nodes at 2 hr as opposed to, e.g. cervical lymph nodes (see Table 4) is similar to that described by others (Griscelli, Vassalli & McCluskey, 1969). The majority of large lymphocytes recirculate poorly from blood to lymph and therefore those in thoracic duct lymph are mainly recently formed in the drainage area, i.e. mesenteric nodes and gut associated lymphoid tissues. One suggestion is that lymphocytes, particularly lymphoblasts, show some degree of recognition for and thus preferred migration to their tissue of origin (Rose, Parrott & Bruce, 1976). Another possible explanation is that this early high concentration in the mesenteric node is due to substantial localization of cells in the lymph node following extravasation in the gut and transport up the afferent lymphatics—such a large afferent supply would not be available to lymph nodes in other sites.

It would appear that there is a significant recirculation of lymphocytes through non-lymphoid tissues which although of low volume may be rapid. The functional significance of this migration requires elucidation but it is presumed to be of importance in, for example, the effector limb of the delayed type hypersensitivity response.

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An experimental comparison of radioactive labels with potential application to lymphocyte migration studies in patients

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SUMMARY

The suitability of two radionuclides (^{99m}Tc, ^{111}In) for labelling lymphocytes have been evaluated in rats by comparison with a standard method using ^{51}Cr. For the study of lymphocyte migration in patients labelling with ^{111}In-labelled oxine is clearly the most promising because both *in vivo* and *in vitro* it remains associated with lymphocytes and the labelled cells migrate normally into lymphoid tissues. The physical characteristics of ^{111}In are also favourable. Not only does ^{99m}Tc rapidly dissociate from lymphocytes but also it compromises their ability to recirculate from blood to lymph.

INTRODUCTION

The radioactive labelling of cells has been chiefly responsible for elucidating the migration pathways of lymphocytes in experimental animals. By this and other methods the unique recirculation of lymphocytes has been shown to be central to the unfolding of cellular events in immune responses (Gowans & McGregor, 1965; Ford, 1975). In man the direct study of this function has been very limited because of the lack of a suitable radioisotopic label. Of the many isotopes used in animals, [^{51}Cr]sodium chromate has been the most popular as a lymphocyte label and enough is known of its properties to regard it as a standard against which other radioactive labels can be compared for specific applications. At least two groups have used ^{51}Cr to study lymphocyte migration in patients (Hersey, 1971; Scott *et al.*, 1972). They labelled peripheral blood lymphocytes *in vitro*, re-injected them i.v. and followed their accumulation in certain lymphoid tissues by surface-detection devices. These studies, as well as complementary evidence obtained by injecting ^{3}H-labelled lymphocytes into patients with thoracic-duct fistulae (Revillard *et al.*, 1968), have suggested that the model of the recirculating lymphocyte pool built up from animal experiments is broadly applicable to man. However, more extensive evidence as well as insight into abnormalities of lymphocyte recirculation in disease would be obtained if invasive procedures including thoracic-duct cannulation could be avoided.

Labelling with ^{51}Cr has several serious disadvantages for clinical studies: (a) the low gamma-photon emission cannot usefully be detected by the gamma camera; (b) the half-life of 28 days is unnecessarily long; (c) the uptake of label *in vitro* is low and compensating for this by increasing the concentration of isotope produces cell damage (Doenhoff & Davies, 1976), therefore about 500 ml of blood is necessary to provide enough lymphocytes for surface detection; (d) significant dissociation of label from lymphocytes within 24 hr is apparent.

In the hope that they might be more suitable in the context of lymphocyte studies in patients we have investigated two isotopes (Indium-111 and Technetium-99m), which have already been widely used in

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nuclear medicine because of their desirable physical characteristics. ^{99m}Tc has been reported to be a suitable isotope for following lymphocyte migration in mice (Barth, Singla & Gillespie, 1974), and ^{111}In-labelled oxine has been successfully applied to both experimental and clinical studies of the distribution of polymorphs (Thakur *et al.*, 1976a) and platelets (Thakur *et al.*, 1976b). Therefore these two isotopes were used to label rat lymphocytes and their behaviour on re-injection was assessed.

MATERIALS AND METHODS

Animals. Rats of either sex belonging to the highly inbred AO strain were used.

Cell preparation. Thoracic-duct lymphocytes were collected by the method of Gowans as described by Ford & Hunt (1973). They were resuspended in PBS at a concentration of 3×10^7/ml and layered over 3 ml of Ficoll–Hypaque (5 vol. 14% Ficoll and 2 vol. 45% Hypaque). After centrifugation (1200 *g*, 20 min, 20°C) the interface layer was collected, washed in PBS and resuspended for labelling with ^{51}Cr, ^{99m}Tc or ^{111}In. The Ficoll–Hypaque centrifugation step completely removed contaminating erythrocytes which would otherwise have been labelled with each of the three isotopes. This would have distorted the estimates of lymphocyte localization in the recipients, especially in the blood compartment.

Radioactive labelling of cells. Chromium-51. Lymphocytes were incubated at 10^8/ml in medium RPMI 1640 + 10% foetal calf serum (FCS) with 10 μCi/ml $Na_2{}^{51}CrO_4$ (CJS1P, Radiochemicals, Amersham) for 1 hr at 37°C. Before injection the cells were washed three times and centrifuged through a layer of 50% FCS–PBS.

Technetium-99*m*. A similar procedure was followed for labelling with $Na_2{}^{99m}TcO_4$ which had been eluted with NaCl from a sterile generator (MCC.3, Radiochemical, Amersham). The concentration of isotope was up to 3 mCi/ml. The use of $SnCl_2$ and/or Na_2CrO_4 to increase label uptake as suggested by Barth *et al.* (1974) was avoided because they were found to be excessively toxic to the cells.

Indium-111. ^{111}In (INS.1, Radiochemicals, Amersham) was chelated with oxine as previously described by Thakur, Coleman & Welch (1977). Lymphocytes were incubated with 0·1 to 0·5 μCi/ml in normal saline at 10^8/ml at room temperature for 10 min. A higher dose as specified later was used for external detection studies. They were then washed as after ^{51}Cr labelling.

For comparison labelled cells were heat-killed by incubation in PBS at 10^8/ml for 30 min at 56°C. After each labelling procedure cell viability was assessed conventionally by trypan-blue exclusion which was also applied to check the effectiveness of heat-killing.

Sample preparation and assay of radioactivity. At 2 or 24 hr after the i.v. injection of labelled cells the recipients were killed and large volumes of PBS were perfused through their entire vasculature to remove intravascular label (Rannie & Donald, 1977). Tissues were then removed, weighed and counted in a dual channel (LKB Wallac) gamma-scintillation counter. Leucocyte samples obtained from cardiac blood were also counted. The counts in the tissues were always compared to the radioactivity measured in an aliquot of the injected cells. In other experiments cells were injected i.v. into recipients with thoracic duct fistulae and the draining lymph was subsequently examined for radioactivity associated with cells and supernatant lymph. Recipients were examined at different times with a gamma camera (N.D. Selektronic A/S). Dissociation of isotope from cells *in vitro* was measured by counting cells and supernatants from cultures maintained in RPMI + 10% FCS at a concentration of 5×10^6/ml at 37°C.

RESULTS

Approximately 2% of the ^{99m}Tc or the ^{51}Cr which was added to the incubation mixture was incorporated into the cells after 1 hr at 37°C, whereas more than 85% of ^{111}In-labelled oxine was incorporated after 10 min and this occurred equally as well at 37°C or room temperature, although the uptake was slower at 4°C. Labelling with ^{111}In did occur in RPMI + 10% FCS but was significantly reduced compared to normal saline. With each method thorough washing was found to be essential to remove unbound label.

The distribution of labelled lymphocytes after i.v. injection into syngeneic recipients is summarized in Table 1. In general, ^{111}In-labelled viable cells behaved similarly to ^{51}Cr-labelled viable cells. Of particular significance were the observations that about 10 times as much ^{111}In was found in lymph nodes at 24 hr as at 2 hr after injection, while the lymph-node activity following the injection of dead cells was negligible at both time intervals. The splenic localization following injection of ^{111}In-labelled cells accounted for more than 50% of the injected radioactivity at 2 hr and fell to about half this value at 24 hr which can be confidently attributed to physiological redistribution (Ford, 1975). The radioactivity in non-lymphoid tissues using ^{111}In was low as expected; in particular the hepatic localization at around 10% was much less than when labelled dead cells were injected.

The radioactivity in the plasma and in the urine gives some insight into the fate of each isotope after it has been released from living or dead cells. Of the little ^{111}In which was released only a small amount

TABLE 1. Mean per cent injected dose per whole organ in syngeneic recipients after i.v. injection of labelled lymphocytes

Organs	2 hr		24 hr		
	^{51}Cr	^{111}In	^{51}Cr	^{111}In	^{99m}Tc
Lymph nodes	3·41	2·39	26·5	30·8	3·9
	0·06	0·12	0·11	0·26	
Spleen	47·7	56·2	22·0	31·5	8·1
	1·7	2·6	2·6	5·2	
Liver	11·3	10·8	9·0	9·6	18·9
	37·1	39·9	38·1	53·5	
Kidney	0·28	0·39	0·29	0·30	6·7
	4·8	2·0	5·6	1·8	
Gut	4·5	1·9	2·9	3·6	—
	0·52	0·68	0·27	0·75	
Lung	4·5	4·3	1·8	1·6	0·34
	22·7	23·1	1·5	2·2	
Bone (marrow)	14·7	18·1	2·3	2·1	—
	3·9	3·2	7·2	10·1	
Blood (w.b.c.)	1·3	1·6	1·3	2·3	—
	0·4	1·1	0·23	1·1	
Plasma	0·27	0·44	0·18	0·34	0·76
	4·9	5·7	1·1	1·3	
Total recovery (viable cells injected)	88·0	96·1	66·3	82·1	38·7
Urine	—	—	$\geqslant 10$	$\geqslant 0{\cdot}6$	$\geqslant 25$

Values for heat-killed cells appear under those for viable cells for each tissue. The standard errors about the means (of the order of 10%) have been excluded for clarity. Times are hours after i.v. injection.

TABLE 2. Comparison of isotopes

Isotope	Physical half-life (days)	Suitability for external imaging	Uptake by lymphocytes (%)	Labelling time	Elution in in culture (after 3 hr) (%)	Injected activity recovered in recirculating thoracic-duct cells 0–42 hr afterwards (%)
^{111}In-labelled oxine	2·8	Good	85	10 min	5	45
^{51}Cr	28	Poor	2	1 hr	30	22
^{99m}Tc	0·25	Good	2	1 hr	30	0·6

appeared in the urine; some was present in plasma, and was probably due to avid binding to transferrin (Thakur *et al.*, 1977). By contrast, the released ^{51}Cr was largely excreted in the urine and relatively less was bound to plasma protein. After injection of cells labelled with ^{99m}Tc, the levels of radioactivity in the spleen and lymph nodes were much lower than with ^{51}Cr or ^{111}In, and high levels were found in the liver, kidney and urine suggesting that the cells had been damaged and had rapidly lost their isotope. The almost complete failure of ^{99m}Tc-labelled cells to recirculate from blood to lymph (Table 2) was the most damning testimony to the inadequacy of this tracer. By contrast, both ^{51}Cr- and ^{111}In-labelled cells recirculated well from blood to lymph, each with a peak recovery typically at 18 hr after injection (Ford

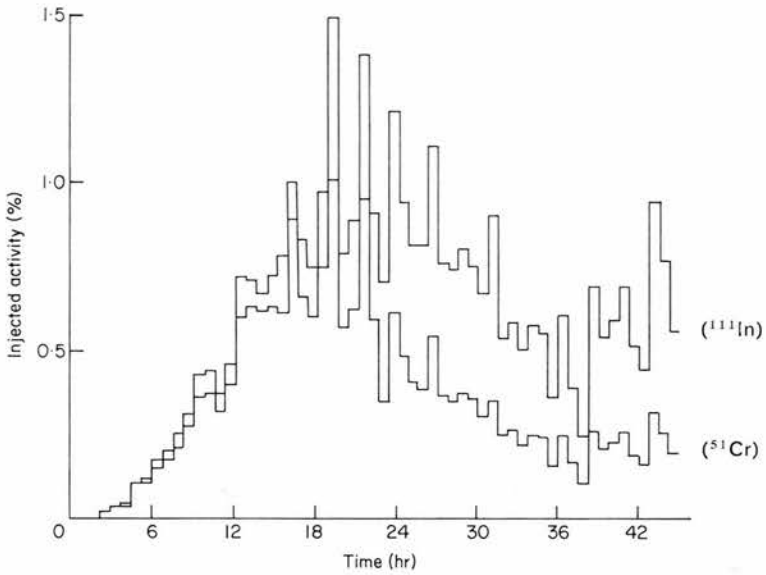

FIG. 1. Recirculation from blood to thoracic-duct lymph of lymphocytes labelled with ^{111}In or ^{51}Cr. Radioactivity was measured in 45 min sequential collections of lymphocytes. With ^{99m}Tc-labelled cells the total recovery in 45 hr was was 0·6% of the injected activity and was too low to plot. The total recovery in 45 hr was 36·04% for ^{111}In, and 21·60% for ^{51}Cr.

& Simmonds, 1972) (Fig. 1). There was a progressive divergence between the recoveries of ^{51}Cr- and ^{111}In-labelled cells in favour of ^{111}In which we attribute to the greater rate of dissociation of ^{51}Cr from cells *in vivo*. The very low rate of elution of ^{111}In, from lymphocytes was supported by the recovery of 36–44% of the radioactivity from thoracic-duct lymphocytes within 45 hr of injection, and was further confirmed *in vitro*, since the loss of isotope from lymphocytes over a 3 hr period in culture was only 5% compared to the spontaneous release of about 30% ^{51}Cr or ^{99m}Tc under the same conditions.

Gamma-camera images were obtained from rats which had received 300 μCi of ^{111}In associated with between 5×10^6 and 5×10^8 cells. At 24 hr after i.v., injection groups of lymph nodes weighing approximately 100 mg could be discerned (Fig. 2 and 3). The images produced by the spleen and the liver were contiguous, but especially at a few hours after injection, when the splenic localization was maximal (Table 1) and the spleen image was more intense.

DISCUSSION

It would be of great interest to study lymphocyte localization in those patients in whom altered migration is probably significant. This includes most chronic inflammatory diseases and neoplastic diseases, especially the lymphomas and leukaemias. For clinical application the ideal characteristics of a radioactive tracer for lymphocytes are lack of toxicity, suitability for external detection and a half-life long enough for studies to be made over 1–2 days and short enough to minimize the body burden of radiation. Above all, the radioactivity should not be lost from the cells in such a way as to give a misleading impression of cellular localization. The present results suggest that ^{111}In-labelled oxine is the most promising agent for the study of lymphocyte recirculation in man and confirms its advantages which have been underlined by previous work (Thakur *et al.*, 1976a,b, 1977) on the evaluation and clinical application of labelling other cell types. The close agreement between the localization of ^{111}In- and ^{51}Cr-labelled lymphocytes indicates that ^{111}In is suitable for migration studies and this, taken together with the high specific activity obtainable, the efficient detection characteristics and the near ideal (2·8 days) half-life of this isotope, suggests that detailed kinetic studies in patients will be possible following reinjection of labelled lymphocytes separated from about 50 ml of blood.

Two notes of caution should be sounded. Firstly, a vital factor in obtaining favourable results is the

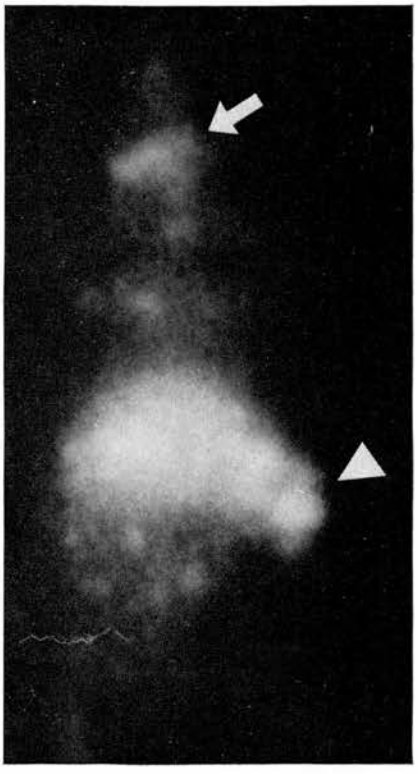

FIG. 2.

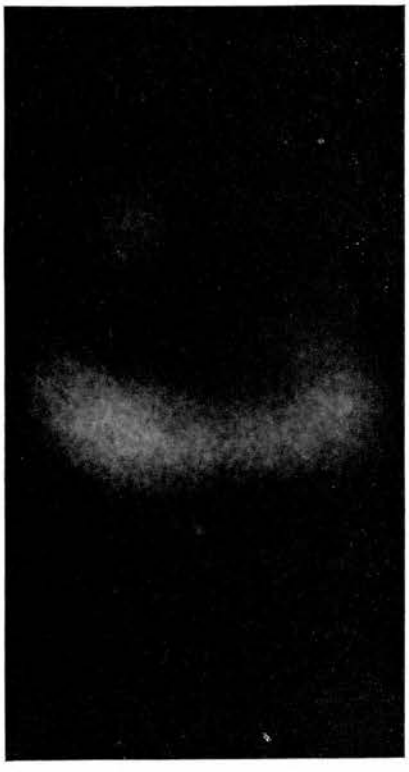

FIG. 3.

FIG. 2. Gamma-camera image of a rat which had received lymphocytes labelled with 300 μCi of ^{111}In 24 hr previously. The large group of cervical lymph nodes (arrowed) as well as other groups of lymph nodes in the thorax and abdomen are visible. The major concentration of radioactivity is present in the liver and spleen. The latter is responsible for the small area of intense activity indicated by the triangle.

FIG. 3. Gamma-camera image (using a pin-hole collimator) of the neck region of a rat which had received lymphocytes labelled with 300 μCi of ^{111}In 24 hr previously. The major concentration of radioactivity is over the superficial cervical lymph nodes. The group of nodes on each side weighs about 100 mg. (Magnification $\times 3$.)

urity of the labelling material. Preliminary trials with ^{111}In produced by α-particle bombardment of a lver target (in contrast to a cadmium target used by the Radiochemical Centre) suggests that certain npurities not only reduce the efficiency of labelling but also impair the migratory function of labelled mphocytes. Chemical instability or impurity of the oxine are other uncertain factors.

The second point concerns the possibility of long-term radiation hazards to patients. Radioactive belling of lymphocytes above a certain intensity will inevitably give rise to radiation damage of these xceptionally radiosensitive cells. Rannie (unpublished) found that labelling thoracic-duct lymphocytes ith ^{111}In at 200 μCi/ml almost completely abolished their capacity to mount a graft-*vs*-host (GVH) eaction as assessed by the sensitive popliteal lymph-node assay (Ford, Burr & Simonsen, 1970). This as hardly surprising since labelling lymphocytes with ^{51}Cr at 100 μCi/ml (producing only about 1% f the radioactivity in the cells compared with the above situation) markedly impaired GVH activity by ne same test (Rolstad, personal communication). The clinical relevance of radiation damage to a small andom sample of the whole-body lymphocyte population lies not in the probable death of these cells but n the possibility of inducing neoplastic transformation. However, we are unaware of any evidence that eripheral lymphocytes as obtained by blood sampling are exceptionally liable to radiation-induced alignant change. The most pertinent information is derived from studies of patients who have been reated by intra-synovial injections of ^{90}Y or ^{198}Au for rheumatoid arthritis of the knee joint which leads

to an accumulation of the isotope in the draining lymph nodes (Stevenson *et al.*, 1973). These nodes (and therefore recirculating lymphocytes) were subjected to intense local irradiation, as witnessed by the high proportion of chromosome aberrations found in the blood lymphocyte population. So far, this procedure has not been associated with any serious consequences (Dolphin, 1973). It should be noted that the size of the labelled sample that is proposed ($50–100 \times 10^6$ lymphocytes) is only about 0·1% of the recirculating lymphocyte pool.

In experimental animals, labelling lymphocytes with ^{111}In has, at present, the practical disadvantages of a short half-life, necessitating weekly or fortnightly orders of ^{111}In, and the lack of a source of ready-made ^{111}In-labelled oxine complex. However, ^{111}In labelling, being potentially more sensitive than ^{51}Cr labelling, might be indispensable for the study of a subset of lymphocytes when only a small number of cells are available for labelling, and then only a minority localize in the compartment of interest, such as a skin lesion. As with ^{51}Cr, autoradiography of ^{111}In-labelled cells is feasible by exploiting the low-energy emission attributed to internal conversion. Such autoradiographs of TDL have suggested that all cells are labelled with ^{111}In with no more heterogeneity of labelling intensity than is found with ^{51}Cr labelling. For labelling target cells in cytotoxicity assays the slow spontaneous release of ^{111}In *in vitro* would be a great advantage. Unless unforeseen difficulties arise ^{111}In labelling would appear to have widespread experimental as well as clinical application.

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PHYSIOLOGY OF LYMPHOCYTE RECIRCULATION IN ANIMAL MODELS

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Several lines of evidence presented at a symposium in Paris in 1968 (1) supported the idea that the dynamics of lymphocyte migration in man was similar in broad principle to the situation which had been established in several other mammalian species in particular the sheep, the calf and the rat. This notion remains valid although a good deal of new information on lymphocyte migration has been forthcoming in the intervening years. The extension of knowledge in this field has mainly been attributable to animal experiments because there has been a lack of technical advances in methods of studying lymphocyte migration in patients. The purpose of this communication is to review briefly some recent advances in lymphocyte recirculation kinetics in the context of possible application to studies in patients. Clinical information of this kind may help to elucidate the cellular events in transplantation and other immune reactions. The functional significance of lymphocyte recirculation has been reviewed previously (2).

A simple description of the concept of lymphocyte recirculation is given in Figure 1. This serves to make a number of basic points as follows. Firstly lymphocytes are unique cells in that they leave the blood to enter tissues and subsequently return to the blood; what is more they follow this circuit many times. Other leucocytes of course enter tissues but they do not return to the blood regularly and in large numbers. Secondly each organ or tissue is an individual case with respect to its capacity to support the migration of lymphocytes and the routes of entry and return may be structurally different in different organs. Thirdly while B and T lymphocytes leave the blood by crossing the same vascular endothelium they later segregate and take divergent routes before returning to the blood. A related question concerns the capacity of subpopulations of B and T lymphocytes to recirculate. In the case of the subpopulations which carry immunological memory the evidence is clear that both B and T memory cells are present in the recirculating pool as are virgin T cells. The evidence as to whether other subpopulations, such as virgin B cells and suppressor cells, recirculate is not yet adequate to give a definite answer (2).

A fuller description of lymphocyte migration kinetics is provided in Figure 2 and Table 1. The Figure is a simplified "wiring diagram" in which the blood compartment is intentionally given a central position. Table 1 gives estimates of a) the flux of small lymphocytes exchanging between the blood and a number of tissues in terms of cells per gm per hour. b) the modal transit time of T lymphocytes migrating through the tissue and c) the content of recirculating small lymphocytes which is of course partly dependent on parameters a) and b). Some of these estimates can be stated with confidence e.g. the modal transit time through skin (blood→ →skin → peripheral lymphatic) of 1-3 hours. The model presented in Figure 2 and Table 1 is eclectic, that is it is based on data drawn from several sources. Most of the recent data is from a series of experiments in which thoracic duct lymphocytes were labelled in vitro with ^{51}Cr and injected i.v. into syngeneic recipients. The number of cells in many different tissues at a range of time intervals after injection were estimated by organ counting and checked by autoradiography. The recipients were killed and their vasculature was perfused with 300ml. of heparinized PBS before samples of the tissues were removed. This removed intravascular lymphocytes and also some of the free label which had eluted from the cells (3).

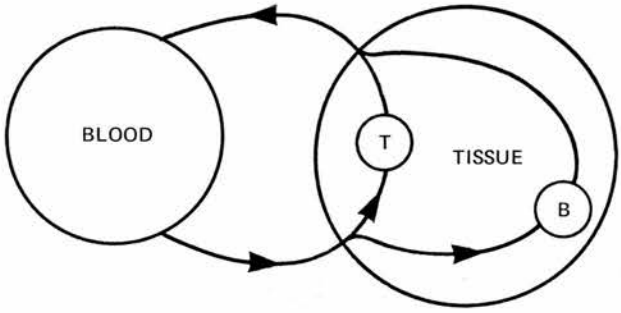

FIGURE 1

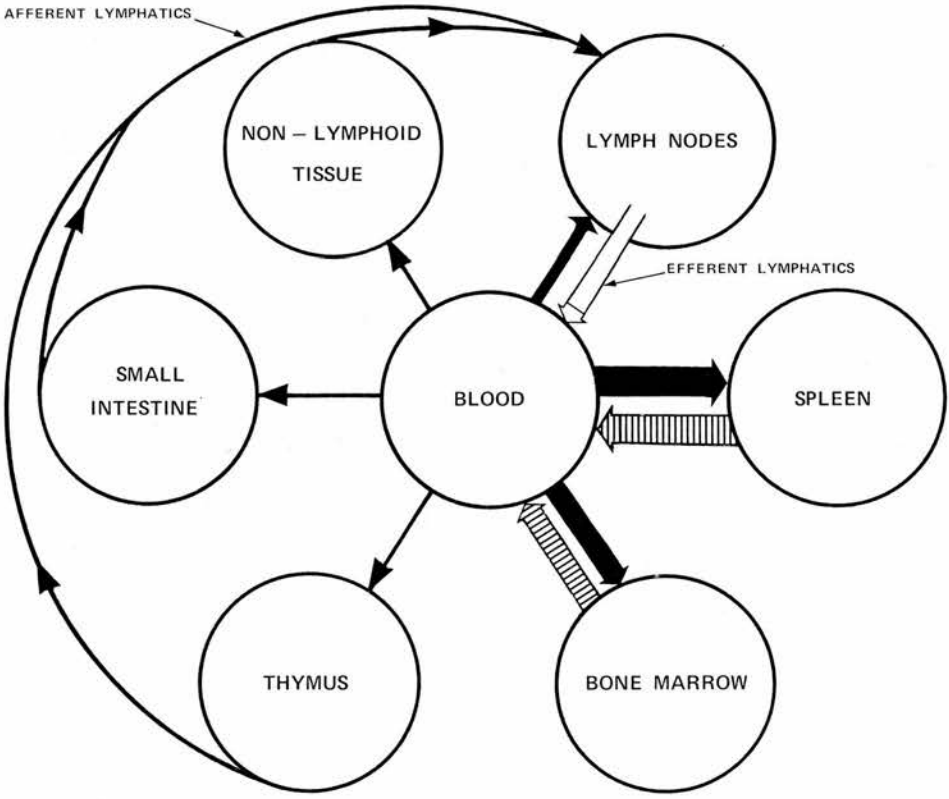

FIGURE 2

TABLE 1

Tissue	Lymphocyte flux x 10^6/g/h	Modal transit time (h) for T cells	Content of recirculating lymphocytes x 10^6/g	[4]Aprox. wt. of tissue g
Spleen	210	4-5	1200	Ca0.4
Lymph-nodes	75	15-18	1300	Ca0.75
[1]Bone (marrow)	2	3-4	6	10
Blood	12	0.4	5	15
[2]Lung	?	? (mins)	45	1.2
Liver	?	?	23	10
Thymus	0.12	Ca.18	2	0.3
[3]Small-intestine	0.6	?	8	5
Skin	0.05	Ca.1-3	0.15	30
Muscle	0.03	"	0.09	100
Ovary	0.25	"	0.75	0.10
Uterus	0.08	"	0.25	0.4
Brain	0.01	"	0.03	1.5

[1] The data refer to whole bone. The great majority of lymphocytes recirculate within the marrow.
[2] Since the lymphocytes in lung may not be extravascular the concepts of flux and transit times may be rather meaningless.
[3] Data refer to the migration of small lymphocytes into the small intestine less Peyer's patches.
[4] Approximate weight of tissue in a 200 g female rat. A major source of uncertainty is the great variability in the spleen and lymph-node weights. A very approximate estimate of the flux and content per whole organ can of course be obtained by multiplication.

MAJOR SITES OF LYMPHOCYTE RECIRCULATION

a) The spleen

The spleen maintains a greater flux of lymphocytes (per gram) than any other organ. In the rat somewhat more than half of the lymphocytes leaving the blood enter the spleen and of these the great majority enter the white pulp. The transit time of T lymphocytes from blood to spleen to blood is rather short with a mode of 4-5 hours and therefore the splenic content of recirculating lymphocytes accounts for only about 20% of the whole recirculating pool (3, 4, 5). Both B and T cells enter via the marginal zone but the former take a more circuitous route including the lymphocyte corona around germinal centres (6). The average transit time of B lymphocytes is several times longer than that of T lymphocytes and in consequence the B lymphocyte content of the spleen is greater than that of T lymphocytes. Neither cell returns to the blood by long lymphatic channels since the isolated spleen releases large numbers of cells into the venous effluent (4). The marginal zone bridging channels (7) carry at least some lymphocytes back into the venous

sinuses of the red pulp. At present no way is known of modifying lymphocytes so as to abolish their capacity to migrate into the spleen; although dextran sulphate administered i.v. reduces the number of lymphocytes entering the spleen compared to the blood level the inhibitory effect on lymph-node localization is much greater (8).

b) The lymph-nodes

The flux of lymphocytes through lymph-nodes is less than half that through the spleen but the transit time of T cells is 3-4 times longer so that content of recirculating lymphocytes is greater (both comparisons per gm. of tissue) (9). There is a good deal of evidence for the longer transit through lymph-nodes but perhaps the simplest is obtained by counting the two organs at 2 hours and 24 hours after injecting radiolabelled lymphocytes. Whereas the splenic activity regularly falls by a factor of between 2 and 4 the lymph-node activity rises by a factor of between 3-8. This divergent trend has been noted after applying several different labels (10, 11, 12). Both B and T lymphocytes enter lymph-nodes by traversing the specialized vascular endothelium in post-capillary venules within the deep zone of the cortex. They subsequently segregate into characteristic areas and eventually return to the blood (6). In the rat at least, there is strong evidence that lymphocytes return exclusively via efferent lymphatic channels; the direct entry of lymphocytes into blood vessels within lymph-nodes does not occur at least within the rat mesenteric lymph-node (13). Several means are available for reversibly inhibiting the entry of lymphocytes into lymph-nodes including treatment of the cells with trypsin (14), papain or sodium azide (15) and treatment of the recipients with dextran sulphate (8, 16).

c) The bone-marrow

It has been realized for some time that the bone-marrow has a small content of recirculating lymphocytes. Recent kinetic studies of transfused, ^{51}Cr labelled lymphocytes has revealed that the flux of recirculating lymphocytes through bone-marrow is substantial and the transit time at 3-4 hours is even shorter than that of the spleen (3, 17). The weight of marrow tissue is several times that of the spleen or of the combined lymph-node mass. Thus the flux per gm is much less than that through the spleen but the flux through the whole marrow is not a great deal less. The functional significance of lymphocyte recirculation within the bone-marrow is probably the same as in other tissues, that is it extends the pool from which antigen-reactive cells can be recruited by antigen. This has been supported by a recent study of a systemic graft-versus-host reaction initiated by the i.v. injection of parental strain lymphocytes into F_1 hybrid recipients. Antigen-reactive parental strain cells were sequestrated in bone-marrow and by 24 hours had transformed to blast cells (17). It seems likely, although it has not been proved, that an immune response can proceed from alpha to omega entirely in the marrow. The anatomical route followed by lymphocytes within the marrow is unclear but it seems likely that they cross a sinusoidal vascular endothelium (18) and later re-enter the blood directly since no lymphatics have been described in bone marrow. The release of recirculating lymphocytes from bone-marrow may be especially susceptible to inhibition by corticosteroids (19).

ORGANS WHICH ARE SPECIAL CASES

a) The lung

The high proportion of lymphocytes which are found in the lungs for the first 30-60 min after i.v. injection has been regarded as a reflection of cell damage and thus essentially artefactual. Several recent observations have suggested that on the contrary this early localization of lymphocytes may be a consequence of a physiological property of the pulmonary vascular bed. These include the following -
1) Early pulmonary localization is equally as pronounced if the lymphocytes are injected into the aorta so that they initially pass through another capillary bed (20, 3).
2) Removing the major site into which lymphocytes migrate from the blood by splenectomy substantially increases the numbers localizing in other sites including the lungs (3).
3) Exposure of lymphocytes to concanavalin A in vitro delays their passage through the spleen and lymph-nodes and also prolongs their retention in the lungs (3).

Straightforward autoradiography of paraffin sections is inedequate to pinpoint the location of lymphocytes in the lung; whether or not they remain intravascular is uncertain.

b) The liver

Another prominent site of lymphocyte localization early after i.v. injection is the liver but in this case the radioactivity in the whole organ falls slightly to reach a plateau by two hours and remains at about 6-10% of the injected dose up to 24 hours after injection (3, 11). There is no doubt that dead lymphocytes or lymphocytes damaged by a long list of chemical and physical agents (including labelling with excessive radioactivity) are mainly removed by the liver (reviewed in 2). Nevertheless it seems that the liver is a site of normal lymphocyte traffic which is intermediate in scale between the major sites of lymphocyte recirculation and non-lymphoid tissues. This is supported by the observation that hepatic lymph in the sheep conveys a greater flux of cells to the draining node than does other peripheral lymph (22) and that the localization of antibody coated cells in the liver can be reduced by carbon blockade of the recipients but untreated lymphocytes are barely affected by such a manoeuvre (23).

c) The thymus

The thymus is one lymphoid organ which is not a major site of lymphocyte recirculation (24). Most of the lymphocytes in the thymus are produced by local cell-division of precursors originally from the bone marrow. However after injecting recipients with radioactively labelled lymphocytes the migration of cells from the blood can be measured by scintillation counting and detected by autoradiography (3). The flux is less than that into the spleen by a factor of about 500 but is 10 times more than the average flux into a non-lymphoid tissue such as skin (comparisons on a per gm basis). The transit time may be of the same order as that through lymph-nodes because the temporal accumulation of radioactivity resembles that in lymph-nodes. The immigrant cells are not uniformly dispersed but are almost all in the medulla. The content of recirculating lymphocytes in the medulla could account for at least some of the mature B and T cells that are present in the cortisone resistant population. The endothelium of post-capillary venules in normal thymus is not high walled although this may alter in disease (25). It is uncertain by which route recirculating lymphocytes return from the thymus to the blood although a lymphatic route would seem more likely. The route may not necessarily be the same as that taken by virgin T cells leaving the organ.

RECIRCULATION IN NON-LYMPHOID TISSUES

a) Under physiological conditions

The observation that peripheral lymph derived from a number of non-lymphoid tissues such as skin, muscle and bone contains a much lower number of lymphocytes than does efferent lymph from a lymph-node established that lymphocyte recirculation through these tissues is on a much smaller scale than through most lymphoid tissues (22). The flux of lymphocytes into a node via afferent lymph is only approximately 5-10% of the flux of cells from the node in efferent lymph although the weight of non-lymphoid tissue in the catchment area of the node is generally many times greater than the weight of the node itself. Nevertheless at least 10 times as many of those cells bound eventually for efferent lymph leave the blood in the node as leave it in the non-lymphoid tissue. By measuring the localization of i.v. injected ^{51}Cr-labelled lymphocytes in a number of non-lymphoid tissues in the rat the flux of lymphocytes was estimated to be less than the flux into spleen or lymph-nodes by a factor of the order of 10^3-10^4 per gm of tissue (3). This is of the same magnitude as can be estimated from the sheep data (22). In that species it was found that the transit time of lymphocytes migrating from blood to tissue to afferent lymph was substantially shorter than the time for cells to traverse the major route from blood to lymph-node to efferent lymph (26). Of course efferent lymph returns cells directly to the blood (unless a chain of nodes is involved) whereas cells ariving at the node in afferent lymph firstly enter the subcapsular sinus and then traverse the node before emerging in efferent lymph. Another study on the sheep from a different group (27) showed that the transit time through the lymph-node of cells injected proximally into the afferent lymphatic was shorter

than that of cells injected i.v. and therefore arriving via the post-capillary venules. This is presumably because the cells arriving via the afferent lymphatic take a faster route (it may be entirely intralymphatic) although they may have further to travel to the efferent lymphatic as the crow flies.

Because lymphocytes spend only a few hours in non-lymphoid tissues before returning via a peripheral lymphatic and because the flux is so low the content of recirculating lymphocytes per gm of tissue is very low indeed which is obvious from inspection of a histological section of many normal tissues in which lymphocytes are present but are few and far between. It is presumed that lymphocytes enter non-lymphoid tissues by crossing the endothelium of small venules but this has not been defined by autoradiography because of the paucity of labelled cells in these tissues. It is also uncertain whether the lymphocytes which enter non-lymphoid tissue are a random sample of the recirculating pool. In the sheep most of the lymphocytes in afferent lymph are immunoglobulin negative suggesting they are T cells. However they display less activity against alloantigens in an intradermal transfer test than do the T lymphocytes in efferent lymph (28). This raises the possibility that different subsets recirculate a) via post-capillary venules in lymph-nodes and b) via non-lymphoid tissues and afferent lymphatics (29), but this proposition needs much additional support before it is accepted.

The kinetic distribution of ^{51}Cr-labelled lymphocytes was studied in the following non-lymphoid tissues of the rat - skin, muscle, ovary, uterus and brain. There are only minor differences between these tissues in the temporal pattern and flux (per gm) of lymphocyte migration. The brain has the lowest flux but even in this case a very occasional labelled lymphocyte can be found in autoradiographs after transfusion of large numbers of tritium labelled lymphocytes (3). Recently the idea that immune responses can be effective against allogeneic cells which are present in what has been regarded as an immunologically privileged site has been neatly supported by an experiment in which allogeneic lymphoma cells were inoculated into the brains of either untreated recipient rats or recipients which had been rendered tolerant to the alloantigens of the donor strain (30). Only the former survived presumably by rejecting the lymphoma in the intracerebral site. This suggests that even the very low grade traffic of lymphocytes into the brain can be effective in eliminating antigenic cells.

b) Sites of inflammation

Large and small lymphocytes are present in cell-mediated immune (CMI) lesions. Sometimes they are the predominant cell type in the cellular infiltrate but in the particular case of allografts undergoing rejection they are usually a substantial minority. The concentration of recirculating lymphocytes in a tissue could increase because of an increased influx from the blood or because of prolonged or indefinite retention within the lesion. There is no doubt that lymphocytes enter CMI lesions at an increased rate as has been shown by an increased localization of labelled cells injected i.v. (31) and an increased traffic of cells in the peripheral lymphatic draining the lesion (32). The recirculating cells are probably retained for a longer time and the subset of antigen specific cells are probably immobilized indefinitely as shown most clearly using grafts of a sponge matrix inpregnated with allogeneic fibroblasts (33).

Two observations suggest that lymphocytes may enter CMI lesions by the same mechanism as they selectively enter lymph-nodes 1) Endothelial cells in the small venules within an adjuvant granuloma (32) or in peroxidase arthritis (34) are plump with large nuclei resembling the high endothelium in the lymph-node paracortex 2) Trypsin treatment of lymphocytes inhibits their migration into lymph-nodes and inflammed skin although entry into other sites including normal skin is unaffected (31). The key to the increased rate of migration is thus an acquired change in the functional properties of vascular endothelium but the stimulus to this change and specifically whether a lymphokine is involved is completely unknown. Between 24 hours and 48 hours after grafting there is a spectacular increase in lymphocyte traffic through an allografted sheep kidney greatly exceeding that through a syngeneic graft (35) suggesting that "recognition factors" liberated following the encounter between virgin lymphocytes and alloantigen may be able to influence vascular endothelium. A lymphokine is by strict definition a product of a sensitized T lymphocyte and is therefore an unlikely candidate in this situation.

CONCLUSION

Although the kinetics of lymphocyte recirculation through different compartments can now be described in some detail little is known of the factors which control the process and may be responsible for alterations in disease. A deeper understanding of the controlling factors and development of the agents which affect the distribution of lymphocytes may afford the possibility of manipulating immune responses. The recent introduction of radiolabelling cells with ^{111}Indium-oxine (36, 37) may possibly be modified in order to follow the distribution of lymphocytes in patients (12). Such a diagnostic tool would be an almost essential step in achieving some practical means of influencing the fate of lymphocytes in patients.

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